

Reproductive ecology and life history trade-offs in a dimorphic polygynous mammal, the New Zealand fur seal

*Thesis submitted in partial fulfilment of the
requirements for the degree of*

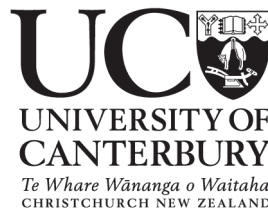
Doctor of Philosophy

at the

University of Canterbury

by

Sandra S. Negro



2008

ACKNOWLEDGMENTS

This study has been funded by the University of Canterbury, the Université de Liège, and Education New Zealand. Each Chapter has a specific Acknowledgment section.

My supervisors, Professor Neil Gemmell and Dr Abigail Caudron were of precious help throughout my training. I learnt a lot from their knowledge and gratefully appreciated all the advice that was given to me. They were supportive in the difficult moment I experienced. I am thankful for the opportunity they gave me to work on the New Zealand fur seal and sea lion.

Thank you to Drs Melanie Pierson, Maxine Bryant, and Andrew Bagshaw for peer-reviewing my manuscripts and for their help in the laboratory. Thank you to Kathryn McBride for her assistance in the laboratory. Thank you to Dr Tammy Stevees for her precious advice for poster and oral presentations. Thank you to Dr Bruce Robertson for his help in the laboratory and advice for poster and oral presentations. It was nice having Sharyn, Genevieve, Laura, Chris, Jonci, Antoine, Manu, Jawad, Daniel, Cécile, Fabrice, Amy, Iris, Thorsten, Lisha, Angie, Wiebke, Maggie, Linda, Joe, Josie and Alex in the Molecular Ecology Group.

Thank you goes to Dr Brigitta Kurenbach for picking me up each lunch and reminding me to eat when I was overwhelmed with work. The hikes, cooking dinners, and cultural activities with her were always enjoyable. Brigitta also peer-reviewed several manuscripts and I am grateful for her useful comments.

My special thank you goes to Margaret Will for her support, useful discussions and help in the laboratory, and advice throughout my PhD. I am grateful for the time she spent reviewing my manuscripts. Outdoor adventures were hilarious and unforgettable. She is a close friend and important to me.

My warmest thank you to my parents, my brother, and my sister for their support and their all year round joyfulness. Every moment with them made me laugh.

The last, but not the least, Jonathan Mottura is my everything. At times he is my confidant and my reason, at others he is my nurse and forces me to eat. He is a very sensitive partner who supports me. His graphic design skills were very useful for the presentation of this work to conferences.

TABLE OF CONTENT

Acknowledgments.....	2
Table of content	3
Thesis abstract.....	6
1 Introduction and literature review	10
1.1 Thesis aims.....	10
1.2 Pinniped Taxonomy	11
1.3 Pinniped ecology.....	12
1.3.1 Abundance	12
1.3.2 Distribution	13
1.3.3 NZFS – abundance and distribution	17
1.3.4 Reproductive ecology - mating systems	18
1.3.5 Reproductive ecology - evolution of polygyny	19
1.3.6 Reproductive ecology – lactation strategies	24
1.3.7 Foraging	25
1.3.8 NZFS – foraging and diet	25
1.4 Molecular analyses in mating system studies	28
1.5 Pinniped life history	29
1.5.1 Life history trade-offs	30
1.5.2 Costs versus benefits of reproduction	31
1.5.3 Comparing males and females	32
1.6 Endocrine systems and reproduction	32
1.7 parasites of pinnipeds.....	34
1.7.1 NZFS - parasites	38
1.8 Brief discussion of the project goals.....	38
2 Paternity testing in open systems and an evaluation of the effects of user-defined parameters on two parental allocation programmes, CERVUS and PASOS, using simulated genotypes.....	43
2.1 Introduction.....	43
2.1.1 Parental allocation programme	45
2.2 Methods.....	49
2.2.1 Simulated Genetic data	49

2.2.2	User-defined parameters and paternity assignment	50
2.3	Results.....	52
2.3.1	User-defined parameters	52
2.3.2	Paternity assignment	53
2.4	Discussion	62
2.4.1	Confidence interval	64
2.4.2	Guideline summary	64
2.5	Acknowledgments.....	65
3	Biases in parentage assignment: do heterozygous males achieve greater reproductive success?.....	68
3.1	INTRODUCTION	68
3.2	METHODS	71
3.2.1	Genetic diversity and rare alleles	71
3.2.2	Simulated genotypes	71
3.2.3	Parentage programmes	75
3.2.4	Assignment analysis by CERVUS and PASOS.....	75
3.3	RESULTS	76
3.3.1	Genetic diversity and rare alleles	76
3.3.2	Assignment analysis by CERVUS and PASOS.....	78
3.4	DISCUSSION	84
3.5	ACKNOWLEDGMENTS	87
4	Alternative mating tactics in the New Zealand fur seal <i>Arctocephalus forsteri</i> : When non-territorial males are successful too.....	90
4.1	INTRODUCTION	90
4.2	METHODS	92
4.2.1	Study site.....	92
4.2.2	Behavioural sampling	92
4.2.3	Male size	94
4.2.4	Genetic sampling	94
4.2.5	Genotyping.....	95
4.2.6	Identity Checking.....	95
4.2.7	User-defined parameters and Paternity Allocation	99
4.2.8	Statistical analysis	100

4.3	RESULTS	101
4.3.1	Male Behaviour:.....	101
4.3.2	Characterization of microsatellite loci	104
4.3.3	Reproductive success of study males.....	104
4.4	DISCUSSION	107
4.5	ACKNOWLEDGMENTS	110
5	Intra-sexual differences in testosterone levels and parasitism in a dimorphic polygynous mammal, the New Zealand fur seal.....	113
5.1	introduction	113
5.1.1	Endocrine – secondary sexual characters interactions.....	114
5.1.2	Endocrine – sexual and aggressive behavioural interactions.....	117
5.1.3	Endocrine – immune system interactions	118
5.1.4	Endocrine – parasite interactions	119
5.1.5	Endocrine – nutrition interactions.....	120
5.1.6	Parasites – social status interactions	120
5.1.7	Parasite – secondary sexual characters	121
5.1.8	Testosterone and costs of reproduction.....	121
5.2	material and methods	124
5.2.1	Study site.....	124
5.2.2	Behavioural sampling	124
5.2.3	Testosterone assays.....	125
5.2.4	Parasitic load and species richness in faecal samples.....	127
5.2.5	Statistical analyses	128
5.3	results	128
5.3.1	Behavioural sampling	128
5.3.2	Testosterone assays.....	131
5.3.3	Parasitic load and species richness in faecal samples.....	133
5.4	Discussion	137
5.5	Acknowledgments.....	142
6	Summary and conclusions	145
6.1	Perspectives.....	147
	References.....	149
	APPENDIX.....	165

Thesis abstract

Reproductive ecology and life history trade-offs in a dimorphic polygynous mammal, the New Zealand fur seal (NZFS)

Sandra Negro

Supervisors: Prof. Neil Gemmell and Dr. Abigail Caudron

Polygyny is the most common mating system in mammalian species (95%), yet our understanding of polygynous systems and microevolutionary processes is still limited. Pinniped mating systems range from extreme polygyny (e.g. elephant seals) to sequential female defence by males and hence have often been used as models for mating system studies. Parentage analysis has enabled the examination of mating success, the identification of pedigrees, and the elucidation of social organisation, greatly enhancing our understanding of mating systems (Chapter 1). However, such analyses are not without pitfalls, with erroneous assignments common in open systems (i.e. when parental and offspring samplings are incomplete). We investigated the effects of the user-defined parameters on the accuracy of parental assignment using two commonly used parental allocation programmes, CERVUS and PASOS (Chapter 2). We showed that inaccurate user-defined parameters in CERVUS and PASOS can lead to highly biased output e.g. the assignment rate at 95% CL of offspring with a sampled known mother to sampled males decreased from 58% to 32% when the proportion of candidate males sampled in the parameter options decreasing 4-fold. We found that the use of both CERVUS and PASOS for parentage assignment can increase the likelihood of correctly allocating offspring to sampled parents to 97% in our study system. Incorrect parental assignment can bias estimates of various biological parameters, such as lifetime reproductive success and mate choice preference, and hence bias ecological and evolutionary interpretations. Here, we propose solutions to increase the power of parentage assignment and hence decrease the bias in biological parameter estimates.

In addition, we analysed the effects of the intrinsic bias in likelihood assignment approaches towards assigning higher probability of parentage on individuals with rare alleles and those with heightened offspring-parent matches, which increase with the number of homozygous loci (Chapter 3). We showed that, as a consequence of the algorithms employed in the programmes CERVUS and PASOS, heterozygote males with rare genotypes are assigned higher rates of parentage than males with common alleles. Consequently, where two males could both be biological fathers of a given offspring, parentage assignment will more often go to the male with the rarer alleles (most often in heterozygous loci). Thus, the commonly used parentage assignment methods may systematically bias the results of parentage analyses towards supporting the notion that females prefer more genetically unusual, most often heterozygous, males. Such a bias may sway investigators towards incorrectly supporting the concept that females choose genetically more unusual males for heterozygosity fitness benefits that underpin the good genes hypothesis, when in fact no such relationship may exist.

In polygynous mammals, successful males mate with multiple females by competing with and limiting the access of other males to females. When the status of many males (age, size, health, genetic etc.) prevents them from achieving the primary mating tactic, theory predicts selection for a diversification of male mating tactics. Recent studies in pinnipeds have shown that observed male mating success was correlated to male paternity success in some species (elephant-seals), but not in others (grey seals). The existence of alternative mating strategies can explain those discrepancies. Chapter 4 implemented the guidelines provided in Chapter 2 and 3 and focused on the polygynous New Zealand fur seal *Arctocephalus forsteri*, predicting that 1) competition for females is likely to cause a diversification of male mating tactics; and 2) that alternative tactics can yield reproductive success. Our results indicated three male behavioural profiles; one corresponded to large territorial males and two illustrated a continuum of alternative tactics employed by non-territorial subordinate males. Our study highlights that holding a territory is not a necessary condition for reproductive success in a population of otariids.

The degree of sexual size dimorphism in polygynous species is expected to increase with the degree of intra-sexual competition and in turn with the degree of

polygyny. The life history of an individual is the pattern of resource allocations to growth, maintenance, and reproduction throughout its lifetime. Both females and males incur viability costs of mating and reproduction. However, male viability costs due to increase growth and male-male competition can be greater than female viability costs of mate choice and reproduction. Although an abundant literature on sexual dimorphism in morphology, physiology, and parasite infections is available, little is known on the intra-sexual differences in physiology and parasite infections associated to the reproductive success of different mating strategies in mammalian species. Chapter 5 examined the reproductive costs between territorial and subordinate males New Zealand fur seal related to their relative reproductive success using a multidisciplinary approach (behaviour, genetics, endocrinology, parasitology). We found that dominant New Zealand fur seal males endure higher reproductive costs due to the direct and indirect effects of high testosterone levels and parasite burdens. Our study highlights that holding a territory confers a higher reproductive success, but induces higher costs of reproduction that may impair survival.

Understanding microevolutionary processes associated to polygynous systems is fundamental in light of the ongoing anthropogenic alteration of the environment through climatic variations and habitat reduction which ultimately affect opportunity for sexual selection and shape the life history trade-offs.

Chapter One

Introduction and literature review

1 Introduction and literature review

1.1 THESIS AIMS

The New Zealand fur seals (*Arctocephalus forsteri*) were commercially exploited to near extinct before they first received protection in 1894 (Lalas and Bradshaw, 2001). Since their protection, *A. forsteri* have re-colonised many areas of their former range. The stability and increasing abundance of the New Zealand fur seal populations provides the opportunity to use this species as a model for investigating polygynous mating systems and microevolutionary processes associated with the life histories of dimorphic polygynous mammals. The overall aim of this thesis was to gain an insight into the diversification of male mating tactics and their reproductive success, and the likely life history trade-offs associated with the primary and alternative male mating tactics.

The first part of the thesis concerns the methods used in parentage analysis. A better understanding of the subtle functionalities of parentage allocation programs and the intrinsic biases in likelihood assignment approaches could help reduce incorrect assignment decisions. The focus of this part of my research was mainly to evaluate:

- 1) The effects of the user-defined parameters on the accuracy of the parental assignment in CERVUS and PASOS, two parental allocation program.
- 2) The bias in parentage assignment methods towards assigning higher probability of parentage on individuals with rare alleles and those with increased offspring-parent matches using CERVUS and PASOS.

The second part of my thesis examines male aspects of the *A. forsteri* mating system and the possible life history trade-offs associated with alternative male mating tactics. A better knowledge of male mating behaviours associated with key constraints (trade-offs) could help us better understand the main factors mediating reproductive success in *A. forsteri* potentially contributing to the long term survival of this and related species by providing information to guide management decisions. The goal of this study was to:

- 1) Implement an improved parentage assignment strategy to the study of male reproductive success in a New Zealand fur seal population
- 2) Describe the diversification of male mating tactics and their associated reproductive success.
- 3) Examine the intra-sexual differences in the costs of reproduction in male fur seals using a multidisciplinary approach that combines behavioural, genetic, endocrinology, and parasitology.

1.2 PINNIPED TAXONOMY

The pinnipeds were long classified as order Pinnipedia which were thought to be separated from, but closely related to the terrestrial carnivores of the order Carnivora. Recent cladistic analyses using both morphological and molecular data clearly support a monophyletic taxon regrouping seals, sea lions, and walruses in the suborder Caniformia of the order Carnivora (Flynn et al., 2005). The monophyletic taxon comprises 33 pinniped species including one species (walrus) of odobenid, 19 species (true seals) of phocids, and 13 species (fur seals and sea lions) of otariids. Numerous morphological and physiological differences exist between otariids (eared seals; fur seals and sea lions) and phocids (true seals). The most obvious morphological differences are the absence of fur and external ears in phocids. The physiological differences of most interest to scientists involve the lactation strategies and unique life histories of these two groups (Riedman, 1990). Phocids are generally larger than the otariids and accumulate higher energy storages before parturition (Kovacs and Lavigne, 1992). Because most phocids forage and reproduce in more variable environmental conditions than otariids, they fast during a short lactation period, while otariids exhibit a more prolonged lactation duration evolving forage cycles (Boness and Bowen, 1996).

The New Zealand fur seal (NZFS) *Arctocephalus forsteri*, is one of the smaller species of fur seal and is found in the southern hemisphere in temperate latitudes around New Zealand, Subantarctic Islands, and southern Australia (see 1.3.3 NZFS - abundance and distribution). *Arctocephalus forsteri* displays natal site philopatry, with

the majority of individuals returning to their birth colony to reproduce (Bradshaw, 1999). For over three decades, *A. forsteri* has been the target of several studies ranging from natural history (Miller, 1971), through history of commercial exploitation and re-colonisation patterns (Bradshaw, 1999, Lalas and Bradshaw, 2001), and more recently, maternal lactation strategies and mating systems (Carey, 1991, Harcourt et al., 1995, Harcourt et al., 2002, Lancaster et al., 2007).

1.3 PINNIPED ECOLOGY

Pinnipeds are large, long lived, aquatic mammals exhibiting delayed sexual maturity and producing generally a single offspring (Bowen et al., 2002). They are marine feeders, but are, in the main, tied to a solid substrate (land or ice) for moulting, mating, parturition, and postnatal pup care (Bartholomew, 1970). The spatial difference between foraging and reproduction location induces a diversification in foraging and reproductive strategies among pinniped species (Kovacs and Lavigne, 1992). Assuming that these characteristics are under selection, variability in foraging success affects survival probability and reproductive performance of individuals. In addition, interactions between individuals and their environment take place at various spatial and temporal scales and influence both the abundance and the distribution patterns of individuals (Bowen et al., 2002).

1.3.1 Abundance

The abundance of a population is determined by births, deaths, and net migration in a population. However, anthropological activities and ecological factors also influence the abundance of a population (Krebs, 2001). Commercial exploitation of pinniped populations dispersed many species and some of them approached extinction (Rodriguez and Bastida, 1998). In the absence of human effects (e.g. disturbance), the combination of ecological factors, such as environmental variability (e.g. El Niño), predation, food supply, breeding habitat and individual choice in breeding behaviour, disease, and competition with other species, regulates the population abundance at a level known as carrying capacity (Read and Le Blanc, 2003). The abundance of pinniped species ranges over 4 orders of magnitude, from the most abundant seal, the

crabeater seals (*Lobodon carcinophagus*), at approximately 12 million, to the Mediterranean monk seal (*Monachus monachus*) at about a few hundred individuals (Reijnders et al., 1993). The accuracy of estimates of abundance vary greatly due to the difficulty in performing good census approaches and the general lack of effort to obtain good estimates, but are important indicators of the status of populations.

1.3.2 Distribution

For a complete understanding of pinniped distribution, a three dimensional view must be considered with the third dimension being the water depth and underlying bathymetry. Pinniped distribution patterns reflect the evolutionary history of pinnipeds, which still need to give birth on solid substrate, but feed in an aquatic environment, and the distribution of these two key resources (Krebs, 2001). Population distribution is affected within these resource constraints by physical (e.g. ice cover), biological (e.g. abundance of predators), characteristics of the habitat (e.g. topography), demographic factors (e.g. population size), morphological and physiological constraints, and human effects (e.g. restriction of habitat availability) (Bowen et al., 2002). The current view of pinniped distribution is incomplete because the census approaches used are based primarily on the location of breeding colonies and predominantly ignore the possibility of pinnipeds foraging at sea or residing elsewhere. For example, pinniped distributions can alter seasonally with changes in pack ice and ice cover for ice breeding species, and those seasonal activities related to synchronized birth, post-natal care, and moult may take place at a different locations than the breeding site (Stewart and Delong, 1993). Overall, there is little to generalise about pinniped distribution, which is patchy and spans estuaries and continental shelves (e.g. harbour seals), deep ocean (e.g. elephant seals), the Arctic (e.g. bearded seals) and Antarctic (e.g. Weddell seals) polar seas, tropical seas (e.g. monk seals), and landlocked seas and lakes (e.g. Baikal and Caspian seals) (Figure 1, 2 and 3).

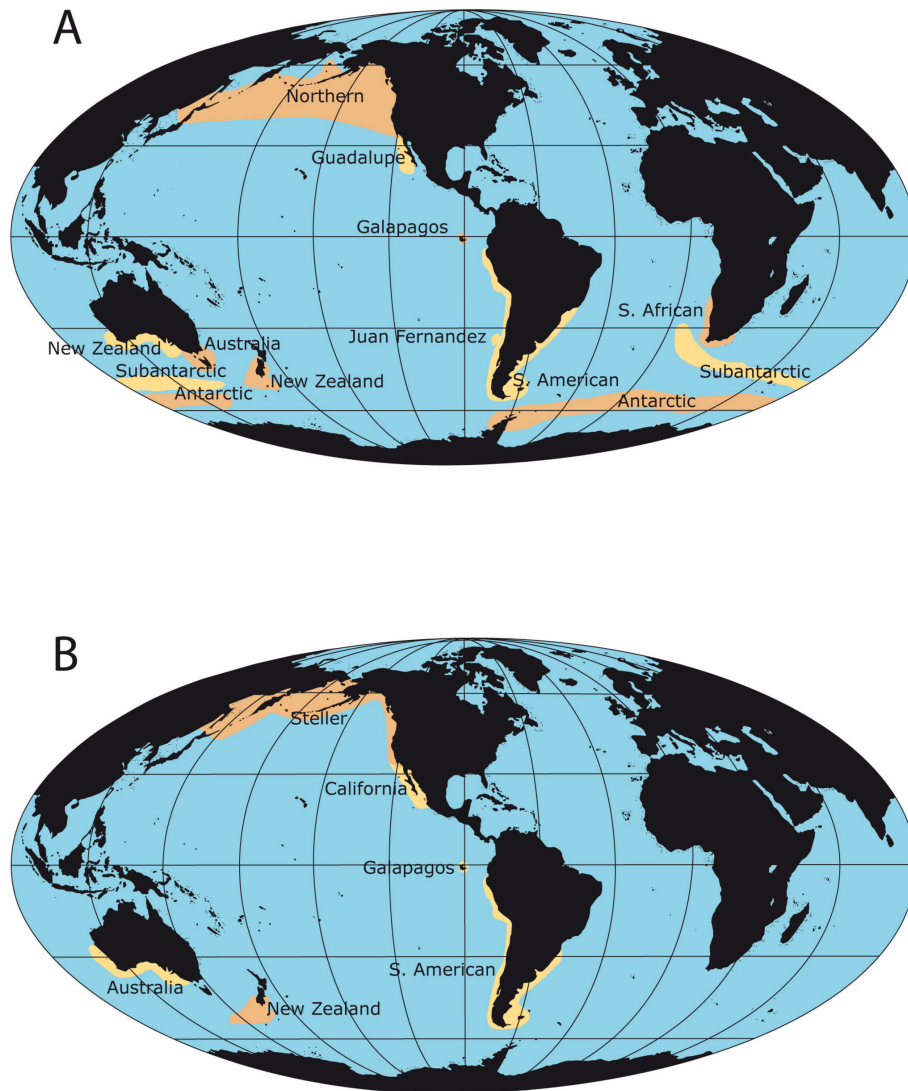


Figure 1: Distribution of fur seals (A) and sea lions (B) based on Riedman (1990).

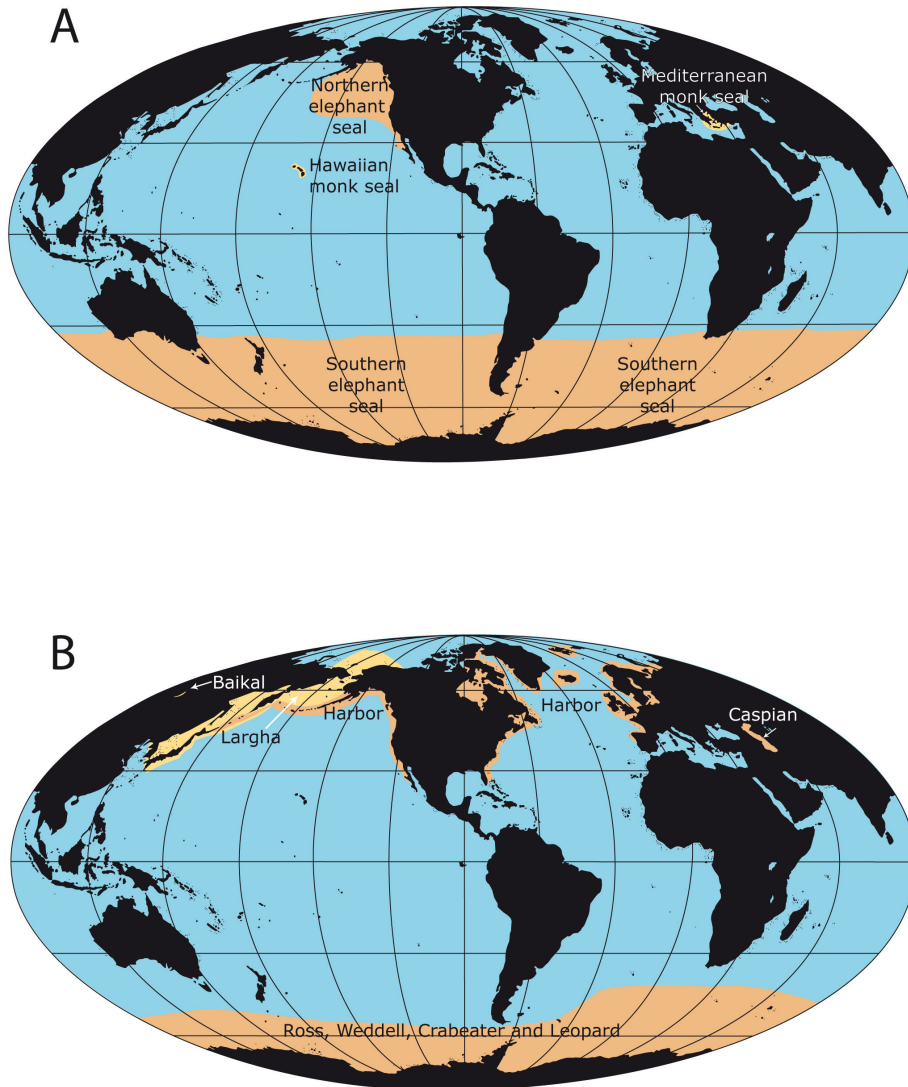


Figure 2: Distribution of monk and elephant seals (A) and some phocid seals (B) based on Riedman (1990).

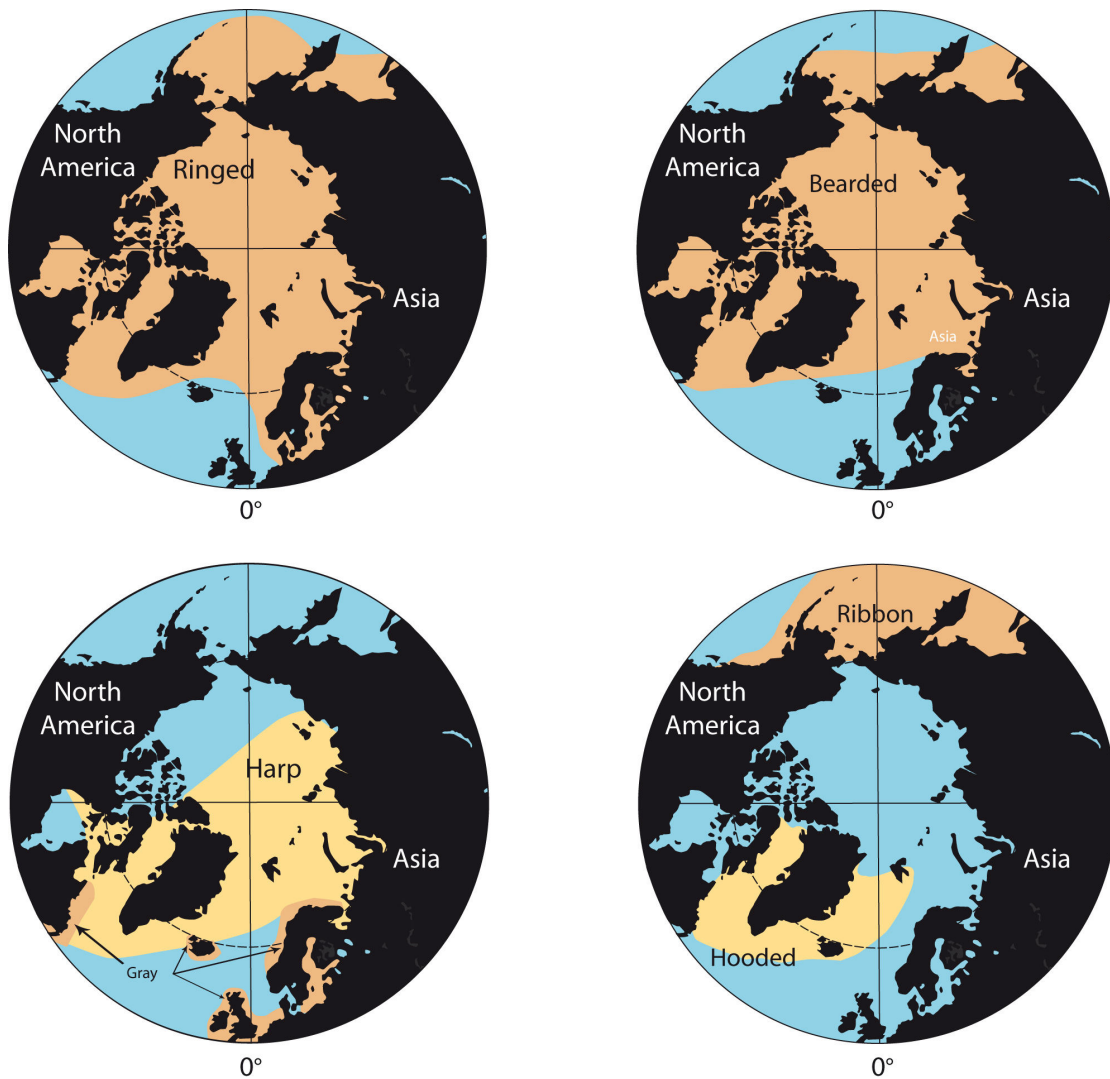


Figure 3: Distribution of Arctic phocid seals (ringed, bearded, harp, ribbon, hooded seals) and gray seals based on Riedman (1990).

1.3.3 NZFS – abundance and distribution

The New Zealand fur seal *Arctocephalus forsteri* (Lesson 1828), is a temperate species whose distribution ranges from the SubAntarctic Islands (i.e. Aucklands, Macquarie, and Campbell) to South Island and the south of the North Island of New Zealand, and the Southern coast of Australia (Figure 1) (Gales et al., 2000, Harcourt, 2001).

Commercial exploitation of pinniped species for meat, oil, fur, and other primary and secondary products was at its peak of activity in the 18th and 19th centuries (Reed, 1965). The New Zealand fur seal reached a critical population level (i.e. hundreds to a few thousands, down from upwards of 2 million animals) in the late 1800's and was first protected from illegal harvest by New Zealand law in 1894, and received further protection under the New Zealand Marine Mammals Protection Act of 1978 (Mattlin, 1987). *Arctocephalus forsteri* has successfully begun to recolonise its pre-exploitation range since its protection (Shaughnessy and McKeown, 2002, Shaughnessy et al., 2005, Boren et al., 2006). The *A. forsteri* population size within New Zealand is estimated to 100,000 individuals and the Australian population size is estimated to 40,000 individuals (Gales et al., 2000, Harcourt, 2001). The current total population size represents approximately 7-10% of the pre-exploitation estimate of 1.5-2 million (Lalas and Bradshaw, 2001). The current general trend of *A. forsteri* population size is increasing. Shaughnessy and McKeown (2002) report a 53% increase in pup production from 1993 to 2000 at the North Neptune Island, South Australia. Exponential increase in *A. forsteri* has been observed in several breeding colonies on the east coast of New Zealand (Boren et al., 2006). Ohau Point seal breeding colony, located 26km north of the Kaikoura township on the east coast of New Zealand, show an exponential growth rate of 32% increase in pup production per annum (Boren et al., 2006). The exponential population growth increases the population density at the breeding sites which may have an impact on the intensity of sexual selection acting through male-male competition and female mate choice. On the other hand, an increase in intra-sexual competition predicts a diversification of male mating tactics (Gross, 1996, Shuster and Wade, 2003).

1.3.4 Reproductive ecology - mating systems

Studies on animal mating systems have brought three generalizations. First, the mating systems of animals are the result of the outcome of the reproductive strategies of individuals rather than the evolved characteristics of species (Clutton-Brock and Harvey, 1978, Gentry and Kooyman, 1982, Rubenstein and Wrangham, 1986). Almost all social relationships lead to intra- and inter-sexual conflict of interest between individuals and in turn lead to various forms of competition and favour generally a compromise between the best mating strategy for males, and the best strategy for females (Davies, 1985). Finally, many mating systems are the consequence of different types of mate guarding which depend on the spatial and temporal distribution of females which in turn depends on the variation in resource distribution (e.g. thermoregulation), predation pressure, costs of social lifestyle, and the activities of other males (Clutton-Brock and Harvey, 1978, Rubenstein and Wrangham, 1986, Carey, 1991).

Pinniped social life holds three principal elements: mother-pup relationships, mating or reproductive systems, and non-breeding aggregations (Caudron, 1997). Pinniped social structures are essentially observed during the reproductive period where a large number of animals aggregate and interact with each other. Three main types of mating systems are defined in vertebrates. They range from monogamous, in which all individuals produce offspring with only one partner during a breeding season (or sometimes during their whole breeding life), through to promiscuous, in which individuals have casual copulations with different partners and produce offspring from several partners during a breeding season, to polygamous, in which individuals of one sex produce offspring with several partners, while individuals of the other sex produce offspring with only one partner over the course of a breeding season (Clutton-Brock, 1989).

Broadly speaking, two pinniped groups can be distinguished: the land-breeding species and the aquatic-breeding species. The land-breeding species include all fur seals and sea lions, northern and southern elephant seals, and gray seals. In a stable and two-dimensional habitat, the aggregation of females has led to the evolution of polygyny and sexual size dimorphism in these species (Weckerly, 1998). The aquatic-breeding species give birth on pack ice, fast ice, or land and mate in the water. This group includes

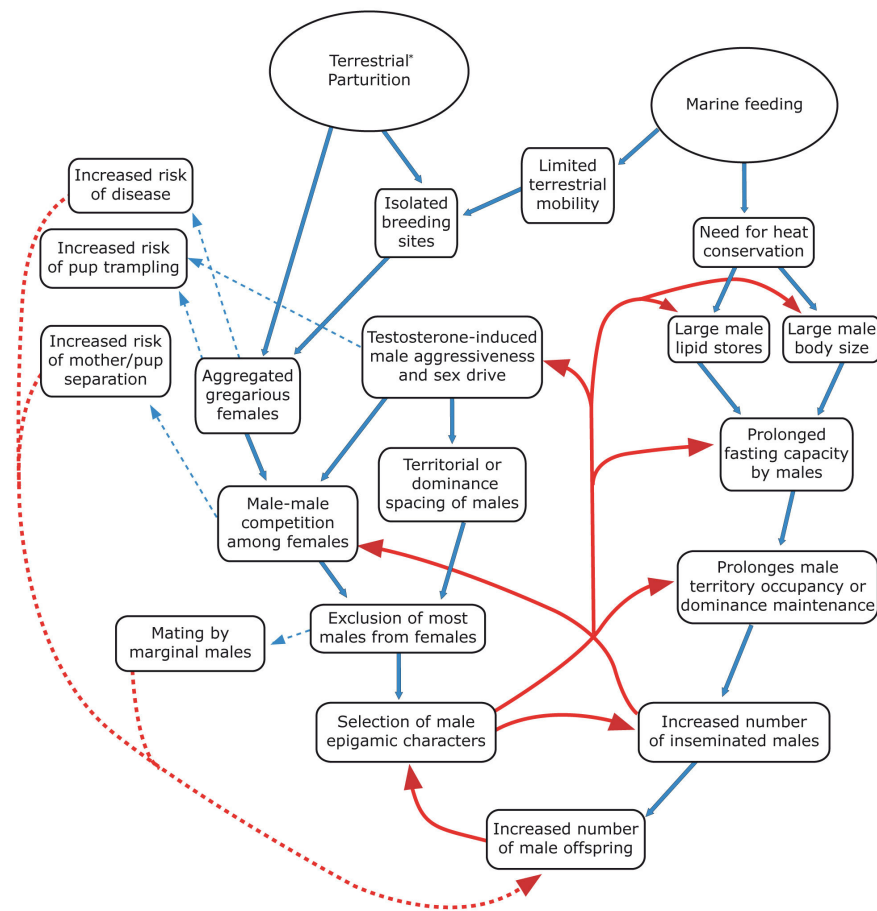
walruses and all other phocid seals, Weddell, Ross, crabeater, leopard, bearded, hooded, ringed, Baikal, Caspian, largha, harp, ribbon, Hawaiian and Mediterranean monk, and harbour (Van Parijs, 2003). In an unstable and three-dimensional environment, females are more widely distributed and the number of females that a male can monopolize is limited. The mating strategies used by ice-breeding species ranges from scrambling where males search for receptive females and progress to the next, through sequential defence where males sequentially defend single female, to lekking where males aggregate and attract females using displays (Van Parijs, 2003).

1.3.5 Reproductive ecology - evolution of polygyny

The fundamental dichotomy in pinniped life history between the marine dependence for foraging and the requirement to come onshore in order to give birth and care for young sets up an evolutionary predisposition for polygyny (Bartholomew, 1970, Boness, 1991b, Le Boeuf, 1991). Bartholomew (1970) proposes a simplified model for the evolution of polygyny in pinnipeds. It involves a complex adaptive suite of traits and behaviours spanning morphology, physiology, ecology, and distribution which has evolved in relation to an amphibious lifestyle (Figure 4).

The degree of polygyny defined as the number of females that a male can monopolize and mate with during the breeding season, is determined by the female distribution during the breeding season and the extent to which a male can limit the access of other males to females. Thus, the topography of the birthing site (including accessibility to water), aquatic versus terrestrial mating, and female intra-specific aggression (hence spacing) are all determinant factors defining the degree of polygyny in pinniped populations (Berta et al., 2006). The degree of polygyny in pinnipeds ranges from extreme (e.g. northern fur seal and elephant seal) where the most successful males may mate with up to 100 females, to moderate (e.g. gray seal, harbour seals) where the most successful males may mate with up to 15 females (Le Boeuf, 1991). All pinniped species giving birth and mating on land are polygynous. Polygynous males show a degree of dimorphism associated with obvious secondary sexual characters. Dimorphic characteristics are the result of sexual selection, acting through male-male competition and female choice, for traits that increase a male's ability to monopolize and defend resources required by

females or the females themselves (Ralls and Mesnick, 2002). Large body size reflects large lipid or energy stores (subcutaneous blubber) permitting dominant males to fast and thus remain ashore during the period covering female oestrus cycles. Extreme sexual size dimorphism is encountered in some pinniped species. The most conspicuous dimorphism occurs in elephant seals. Northern elephant seal males can reach 5-6 times the adult female size and the largest southern elephant seal males may weigh 10 times as much as the adult females (Le Boeuf and Laws, 1994). The elongated proboscis (enlargement of the nasal cavity internally divided by the nasal septum), enlarged canine teeth, and thick cornified skin at the level of the neck of male elephant seals are additional secondary sexual characteristics (Le Boeuf and Laws, 1994). Dominant northern elephant seal males can fast for a period of approximately three months during the breeding season, and this ability to stay on the breeding site for long periods without the need to forage may confer significant breeding advantages to males (Wainstein, 2000). Sexual dimorphism in other species is less pronounced, but still significant. In otariids males are typically 2-3 times the adult female size, and sexually mature males possess huge neck shields with thick fur ruffs (Miller, 1991). Vocal displays (secondary sexual characters) are used in some species of otariids and phocids and play an important role in establishing and maintaining a territory (Slater, 1981, Reby et al., 1999, Kunc and Wolf, 2008).



*Extreme polygyny is not observed in species breeding on ice or in water.

Figure 4: A simplified model of the evolution of pinniped polygyny. The blue arrows correlate the major attributes of typical polygynous mating systems; the red arrows indicate positive (solid) and negative (dashed) feedback loops (modified from Bartholomew, 1970).

In polygynous mammals, male compete for mates either by directly preventing other males from acquiring female (female or harem defence polygyny) or by excluding other males from a essential resource required by females (resource defence polygyny) (Berta et al., 2006). The rookery sites used by land breeding pinniped species (approximately half of pinniped species) are generally located on remote islands or on isolated mainland beaches difficult to access by large territorial predators. Because rookeries are geographically static and the breeding season varies little from year to year, males can anticipate the return of females and compete for territories required by females (in otariids) or establish dominance hierarchies (elephant seals and land-breeding grey seals) prior to the arrival of females on breeding sites (Bowen et al., 2002). On the other hand, females can depend on the predictable presence of birthing sites and on the presence of high quality breeding males (Bowen et al., 2002).

The hierarchical and territorial systems described above are subject to alternative mating tactics by subordinate males (reviewed in Gross, 1996). The diversification of mating tactics are predicted to increase with the degree of polygyny (Shuster and Wade, 2003). In some pinniped species subordinate males can intercept females on their way out to the ocean or back to the colony after a short foraging trip (Lidgard et al., 2004). In other cases, sneaking is used by individual males and implies a subtle and subversive behaviour characteristic (Gross, 1996). Another alternative mating tactic used by non-territorial males involves coalitions of young males around large harems who attempt to distract territorial males to create mating opportunities (Campagna et al., 1988). In this situation, females generally move away from intruders or vocally protest mounting attempts by subordinate males, attracting the notice of alpha males (Boness et al., 1982). An extreme example of such an alternative male mating tactic is observed in southern and Australian sea lions where sub-dominant males coordinate group raids into dominant territories. In this case, the alpha males are unable to deal with all of the raiders simultaneously and some are able to forcibly mate or abduct females (Campagna et al., 1988). In these systems the older or more dominant females generally are located towards the centre of the territories and are more likely to mate with dominant male. Younger or less dominant females are pushed to the edge of the territories and are more exposed to mating attempts by subordinate males. Thus, it is possible that the function of such satellite strategies may be to reinforce tight female aggregations (Bartholomew, 1970; Figure 4).

Other alternatives to the primary dominant mating tactic have been documented. Some authors suggest an active role by female fur seals in mate choice (Heath, 2002, Hoffman et al., 2007). Studying South American fur seals in Peru, Majluf (1987) observed that high temperatures at the breeding sites required females to travel routinely to the water away from pupping sites where copulations may occur, which she suggested is a thermally induced lek. Heath (2002) suggests that the need for routine access to cooling areas (e.g. ocean) has driven the evolution of this alternative mating systems in both South American and Galapagos fur seals. Given the above scenario, the observed male mating success (i.e. copulations) may be unrelated to the number of females in dominant male territories in some species (e.g. South American fur seals).

Arctocephalus forsteri breeds in densely-packed colonies on rocky beaches, backed by steep landscape and exposed to the prevailing winds (Crawley and Wilson, 1976). The mating system displayed by this species is a resource-defence polygyny (Carey, 1991). A study on Macquarie Island reports a high level of hybridization (17%-30%) between Antarctic (*Arctocephalus gazelle*), Subantarctic (*A. tropicalis*), and New Zealand (*A. forsteri*) suggesting that *A. forsteri* utilises alternative mating tactics other than territory defence to achieve paternities (Lancaster et al., 2007). However, no studies have described in detail the full spectrum of alternative mating tactics used by non-territorial males and their reproductive success in *A. forsteri* and other otariid species.

Three additional phocid species give birth on land, but have aquatic mating (harbour seals, Hawaiian monk seals, and Mediterranean monk seals). Few studies have focused on the type of mating system used in these aquatically mating species and all three species are thought to have a low level of polygyny (Coltman et al., 1998, Hayes et al., 2006) . Hanggi and Schusterman (1994) show that male harbour seals perform underwater acoustic displays along routes frequently travelled by foraging females. Alcorn (1984) reports mobbing behaviour in Hawaiian monk seal males attempting to mate with females. Le Boeuf and Mesnick (1991) propose a male-biased sex ratio (between 4:1 and 25:1) to explain this phenomenon.

All ice breeders (approximately one third of pinniped species) have an aquatic mating

system. The degree of polygyny displayed by these species is reduced compared to land breeding species due to the difficulty of monopolizing and restricting access to females in a fluid three-dimensional or in physically unstable environments (Van Parijs, 2003). Several studies have focused on the mating behaviour of ice breeding species (McRae and Kovacs, 1994, Gelatt, 2001, Van Parijs and Clark, 2006, Harcourt et al., 2007, Harcourt et al., 2008). Harp seals and ribbon seals have similar body size of the sexes and are thought to be promiscuous, breeding in largely open floating pack-ice (Van Parijs, 2003). A similar or a slight difference in body size between the sexes might be explained by a low selective advantage for males to be larger than females because of the limited ability of males to monopolize females in such environment.

1.3.6 Reproductive ecology – lactation strategies

Male pinnipeds exhibit no paternal care. Maternal care involves transfer of energy-rich milk to offspring and protection from terrestrial predators and conspecifics (Bowen, 1991). In walrus, females may teach their offspring to forage during their foraging trips and lactation period (Riedman, 1990). The spatial and temporal separation of energy acquisition (aquatic foraging) from high levels of energy expenditure (terrestrial lactation) has driven the evolution of three basic lactation strategies in pinnipeds: foraging cycle (all otariids and some of the smaller phocid species), fasting (the larger-bodied phocid), and aquatic nursing (walrus) (Bowen et al., 2002).

Pinniped females may trade-off investment in current offspring versus investment in future offspring. This trade-off may lead to conflict between female and nursing pup over the level of investment received by the offspring (Bowen et al., 2001). Body size at weaning affects subsequent offspring survival (Craig and Ragen, 1999). Thus offspring nutritional requirements and physiological abilities are important factors in the evolution of lactation strategies. For example, the fasting ability of offspring limits the duration of the foraging cycle in female fur seals and sea lions (Lunn et al., 1993).

Maternal attendance patterns of lactating *A. forsteri* show foraging cycles associated with high frequency of overnight foraging trips (Harcourt et al., 1995, Boren, 2005). However, environmental and climatic factors may play a significant role in shaping the maternal

lactation strategies displayed by *A. forsteri* (Boren, 2005).

1.3.7 Foraging

As pinniped species feed underwater and generally at remote locations, indirect methods must be used to gain insight into foraging behaviour and diets. Foraging is a critical determinant of fitness because successful foraging is essential for survival and reproduction (Bowen et al., 2002). Figures 1, 2 and 3 show the broad distribution of pinnipeds and as a result forage at highly varied spatial and temporal scales, exploiting a wide range of preys (Table 1).

1.3.8 NZFS – foraging and diet

Information on foraging areas of *A. forsteri* has been recorded using satellite telemetry and geolocation. *Arctocephalus forsteri* has been reported in up-welling zones, oceanic frontal systems, and continental shelf-edge regions where primary productivity is high (Arnould, 2002). *Arctocephalus forsteri* diet may vary temporally with the seasonally prey abundance (Table 1.1) (Arnould, 2002).

Table 1: Pinniped species specific prey range.

<i>Species</i>	<i>Food resource</i>	<i>Exemplary food types</i>
Hawaiian monk seal	Fish, cephalopods, other invertebrates	Flatfish, scorpaenids, larval fish, moray eels, conger eels, octopuses, spiny lobsters
Mediterranean monk seal	Fish, cephalopods	Barbouni, gopa, pargo, rays, octopuses
Northern elephant seal	Fish, cephalopods, tunicates*	Pacific whiting, squid, octopuses, pelagic red crabs
Southern elephant seal †	Fish, cephalopods, other invertebrates	Antarctic blennies, squid, octopuses, amphipods
Weddell seal ‡	Fish, cephalopods, krill, other invertebrates	Atlantic cod, squid, octopuses, <i>Euphausia superba</i> , amphipods
Ross seal ‡	Cephalopods, fish, other invertebrates, krill	Squid, octopuses, cuttlefish, <i>E. superba</i> , amphipods, lantern fish, dragon fish
Crabeater seal	Krill, cephalopods, *fish*	<i>E. superba</i> , <i>E. crystallorophias</i> , octopuses
Caspian seal ◇	Fish, crustaceans	Sculpin, gobies, herring
Leopard seal ‡	Birds, pinnipeds, krill, fish, cephalopods, other invertebrates	Penguins, crabeater seals, <i>E. superba</i> , squid, octopuses
Hooded seal	Fish, cephalopods, other invertebrates	Redfish, cod, capelin, herring, squid, octopuses, mussels, shrimps
Bearded seal	Bivalve molluscs, crustaceans, cephalopods, fish, other invertebrates	Clams, gastropods, shrimps, crabs, worms, cod, octopuses
Harp seal †‡◇	Fish, crustaceans, krill	Capelin, cod, herring, crabs, shrimps, euphausiids, amphipods
Ribbon seal	Crustaceans, fish, krill, cephalopods	Crabs, shrimps, mysids, cod, pollock, eelpouts, squid, amphipods
Baikal seal	Fish, crustaceans	Gobies, <i>Comephorus</i>
Ringed seal ‡◇	Fish, shrimps, amphipods, euphausiids	Cod, shrimps, amphipods, euphausiids
Largha seal †	Fish, cephalopods, crustaceans	Cod, rockfish, sculpin, flounder, octopuses, amphipods
Harbor seal †‡◇	Fish, cephalopods, krill, other invertebrates	Herring, cod, flounder, sculpin, gadoids, salmon, octopuses, whelks, shrimps, amphipods
Grey seal ‡◇	Fish, cephalopods, crustaceans	Salmon, cod, herring, mackerel, squid
California sea lion	Fish, cephalopods	Anchovies, herring, Pacific whiting, rockfish, hake, salmon, squid, octopuses
Galápagos sea lion	Fish, cephalopods	Octopuses

<i>Species</i>	<i>Food resource</i>	<i>Exemplary food types</i>
Steller sea lion ‡	Fish, cephalopods, crustaceans, bivalve molluscs, pinnipeds	Rockfish, sculpin, capelin, flatfish, squid, octopuses, shrimps, crabs, northern fur seals
Australian sea lion ‡	Fish, cephalopods, crustaceans, birds	Whiting, salmon, squid, rock lobsters, crayfish, penguins
New Zealand sea lion ‡	Fish, cephalopods, crustaceans, bivalve molluscs, birds	Flounder, squid, octopuses, crabs, mussels, penguins
Northern fur seal ‡◊	Fish, cephalopods, birds*	Pollock, herring, lantern fish, cod, rockfish, squid, loons, petrels
Guadalupe fur seal	Fish, cephalopods	Rockfish ?
Juan Fernández fur seal	Fish, cephalopods, crustaceans	Squid, lobsters
Galápagos fur seal	Fish, cephalopods	Anchovies, mackerel, small squid, lantern fish, deep sea stints
South American fur seal	Fish, cephalopods, crustaceans, other invertebrates	Anchovies, carangids, sea trout, squid, sea snails
New Zealand fur seal ◊	Cephalopods, fish, birds, crustaceans	Octopuses, squid, barracouta, penguins, crabs, rock lobsters
Antarctic fur seal ‡	Krill, fish, *cephalopods, *birds*	<i>E. superba</i> , squid, notothenid fish, penguins
Subantarctic fur seal ‡	Cephalopods, fish, krill, birds	Notothenid fish, squid, euphausiids, penguins
South African fur seal †	Fish, cephalopods, crustaceans, birds	Mackerel, pilchard, maasbanker, anchovies, squid, octopuses, cuttlefish, shrimps, rock lobsters, penguins
Australian fur seal	Cephalopods, fish, crustaceans	Squid, octopuses, snook, rock lobsters, mullet, parrot fish, whiting, barracouta
Walrus	Benthic invertebrates, fish, cephalopods, pinnipeds	Bivalve molluscs (soft shelled clams, cockles, Arctic rock borers), octopuses, polar cod, annelid & sipunculid worms, crabs, shrimps, amphipods, mysids, sea cucumbers, ringed seal
<p>* Known to be a relatively minor food resource.</p> <p>† Diet known to vary with age.</p> <p>‡ Diet shows distinctive geographic variation.</p> <p>◊ Diet known to vary seasonally.</p>		

1.4 MOLECULAR ANALYSES IN MATING SYSTEM STUDIES

Social behaviour covers all interactions between individuals including mating behaviour, family and group interactions, cooperation, competition, and the evolution of societies (Tinbergen, 1953). Molecular approaches have the potential to supplement and refine studies over this entire range. Integrating molecular techniques with field methods in studies of social behaviour has led to a refinement of details, but also has led to contradictions of existing paradigms and new hypothesis (reviewed in Hughes, 1998).

Polymorphic molecular markers offer new opportunities for evaluating fine scale relationships within and between populations. DNA profiling is a commonly used molecular genetic technique based on a very high rate of variation in repetitive DNA regions such as microsatellites and minisatellites. The increasing use of microsatellite markers enables greater insights into mating systems through increased understanding of parentage (Ambs et al., 1999, Gemmell et al., 2001, Foerster et al., 2003, Cohas et al., 2007), social organization (Conrad et al., 1998, Cohas et al., 2006), and sexual selection (Payne, 1979). High-throughput genotyping techniques provide an unprecedented amount of genetic data, which has resulted in a surge of techniques for the analysis of parentage, social organization and sexual selection in natural and experimental populations. Several statistical packages have been developed to extract all embedded information. However, the details and implementation of the diverse techniques often differ in subtle ways that can influence the results and interpretation of parentage analyses and other individual- and population-based analyses (Jones and Ardren, 2003). An important advance in parentage analysis is the use of likelihood statistical approaches to assess confidence in parentage assignments (Marshall et al., 1998, Neff et al., 2000b). Hadfield et al. (2006) develop a class of full probability models that estimate parentage and a wide range of population-level parameters simultaneously by combining behavioural, spatial and genetic data in a Bayesian framework.

One of the factors influencing parentage assignment is incomplete parental sampling which can induce high rates of erroneous assignments, if the number of missing parents cannot be estimated with accuracy (Nielsen et al., 2001, Duchesne et al., 2005). Few methods have been developed that provide unbiased parentage assignment and an

estimation of the relative reproductive success of different groups (Araki and Blouin, 2005, Hadfield et al., 2006). Incorrect assignment decisions can have a large effect on estimates of various biological parameters and hence bias ecological and evolutionary interpretations.

1.5 PINNIPED LIFE HISTORY

The life history of an organism is the product of resource allocation to growth, maintenance, and reproduction throughout its lifetime. The analyses of life histories aim to explain the scheduling of the allocation process throughout an organism's life. The variable "time" is often used to examine variation in resource allocation and the major component of this independent variable is age. Body condition and foraging skill are important variables that affect reproduction and, indirectly fitness (Boyd, 2002).

Most studies of pinniped life history consider age to be the main force driving pinniped life histories. However, age per se may have an insignificant influence on fitness. Pinniped body size has been recognized as a determinant of sexual maturity in pinnipeds and can be expressed as a decreasing function of growth rate. Hence, individuals residing in pinniped populations whose census size lie below the environmental carrying capacity would show a higher growth rates and consequently become sexually mature at an earlier age (Bengston and Laws, 1985).

Three main features characterize pinniped life history: (1) pinnipeds are long-lived (20-40 years) aquatic mammals with high annual survival rates; (2) depending on the species considered, a 2-6 year delay is observed in the average age at sexual maturity; and (3) the litter size is generally limited to a single offspring. Variations in these features at the level of individuals and species provide insight into the evolution of pinniped life histories (Boyd, 2002). The key demographic parameters used so far to describe the life histories of *A. forsteri* are mean adult female body mass (34.32-41.85 kg), pup survival rate (0.40-0.92), mean age at first parturition (females, 4-8 years of age; males, 8-10 years of age), and mean pregnancy rate (0.67) (Wickens and York, 1997, Boren, 2005, McKenzie et al., 2007).

1.5.1 Life history trade-offs

Pinniped life histories have evolved under a combination of factors that are regulated around the need for individuals to balance their energy budgets and involve constraints associated with (1) being warmed-blooded species feeding in water that is 25 times more conductive than air, (2) the high spatial and temporal variability in the aquatic resource distribution, and (3) a terrestrial phase during the reproductive cycle (Boyd, 2002). For example, species that exploit distant, unpredictable food sources necessitate larger body mass than species that exploit closely distributed food sources to the pupping location. This can be explained by the constraints that females must bear, which are associated with the duration a pup can be left without feeding with low risk of starvation. If females cannot forage gainfully during lactation, they must carry with them at parturition most of the food reserves required to raise their pup to independence (Boyd, 1998). Another example is the extremely seasonal food availability in high latitude that has led to extreme spatially and temporally synchronized reproduction (e.g. birth to weaning in hooded seals is approximately two weeks). The highly constrained reproductive period might have led to the evolution of highly competitive, polygynous mating systems (Bartholomew, 1970).

The *A. forsteri* breeding season begins when adult males come ashore and establish territories in spring. Females haul out from mid-November to late December to give birth (Mattlin, 1978, Goldsworthy and Shaughnessy, 1994, Boren, 2005). Females enter in oestrus approximately one week after parturition followed by alternation between foraging at sea and pup nursing onshore. The breeding season ends when all females terminate their oestrous cycle and start their foraging trip at sea in February (Goldsworthy and Shaughnessy, 1994). The exponential growth experiencing by several *A. forsteri* breeding colonies on the east coast of New Zealand may be explained by the proximity to a predictable food source (Boren et al., 2006). The continental shelf is narrow off the east coast of some breeding sites and the steep slope forms a system of trenches that commonly produce upwelling of subsurface waters (Garner, 1953). Mesopelagic fish (e.g. lantern fish) and squid migrate vertically in the water column within the trench at night (Robertson et al., 1978). Population growth increases the number of individuals at the breeding sites which in turn increase the degree of polygyny

(hence the intensity of sexual selection) as the boundary regions of territorial males are topographically demarcated and change little (Miller, 1971, Mattlin, 1978, Carey, 1989, Boren, 2005). One expected outcome of the increase in population density and the associated increase in the degree of polygyny is an increase in the reproductive success of a defined number of individuals that are able to defend resources (i.e. a territory) required by breeding females (Wingfield et al., 1990). However, this heightened reproductive success may come at a cost, as parasitism is also expected to rise in proportion to the population density. Pioz et al. (2008) show correlations between population density and disease in alpine chamois. Zuk and McKean (1996) and Moore and Wilson (2002) identify correlations between male parasitism and male-biased mortality, which is expected to increase with the degree of sexual selection in polygynous species. There are obvious fitness gains from territorial behaviour, but the benefits and costs of reproduction might be different between males using the primary and alternative mating tactics.

1.5.2 Costs versus benefits of reproduction

There are clear fitness gains from reproduction, but despite this individuals may choose not to reproduce annually. However, when individuals do reproduce, they adjust the amount of resources they supply to their offspring. This can be explained by the investment decisions individuals must make each season in order to maximize their fitness across their whole lifetime (Boyd, 2002). Some examples in the literature illustrate the costs and benefits from reproduction. In northern elephant seals, Reiter and Le Boeuf (1991) show primiparity of females at age 3 reduced survival compared to females that reproduce for the first time at age 4. In gray seals, Pomeroy and co-workers (1999) show that females that expend more on their offspring in one year have decreased reproductive success in the following year. Thus, females must optimize the balance between the fitness benefits and costs of reproduction by making decisions on energy allocation between growth, maintenance, and reproduction. In natural populations and variable environments, few individuals may succeed in reaching the optimum, but natural selection favours individuals that make energy allocation decisions that approach the optimum most closely (Boyd, 2002).

1.5.3 Comparing males and females

Mammalogists have given more attention to female than male pinnipeds because females are the limiting sex and because it is more difficult to study male reproductive success. Nevertheless, male pinnipeds may invest considerable amounts of their energy reserves in reproduction. In terms of number of offspring, the potential benefits from reproduction in successful competitor males are greater than for females due to the restriction of producing generally a single offspring per breeding season (Payne, 1979). In general, males have shorter life expectancies compared to females as shown in several studies where the annual survival rates in males are lower (Arnould and Duck, 1997, Wickens and York, 1997). However, little is known about how male survival rates are influenced by the investment in reproduction effort.

The age of physiological maturity in male and female pinnipeds is probably similar (Boyd, 2002). However, many authors distinguish between physiological and social maturity (age at which individuals are capable of competing for breeding). Paternity results from several studies on male reproductive success are shedding doubt to the former definition of social maturity because the pattern of reproductive success in males often deviates from the pattern suggested by the observed social structure.

1.6 ENDOCRINE SYSTEMS AND REPRODUCTION

The endocrine system is built up of small organs located at different regions of the body that involve the release of extracellular signalling molecules termed hormones. The endocrine system is instrumental in regulating a broad range of body functions such as metabolism, growth, development and puberty, tissue function, and behaviour (reviewed in St. Aubin, 2001). The endocrine system is an information signal system using blood vessels as transportation route. Hormones are released into the bloodstream by specialized cells that are usually located in small glands (St. Aubin, 2001). Their great potency and ability to broadly influence body functions are associated with a fine set of negative and positive feedback loops that may link several glands (Figure 5).

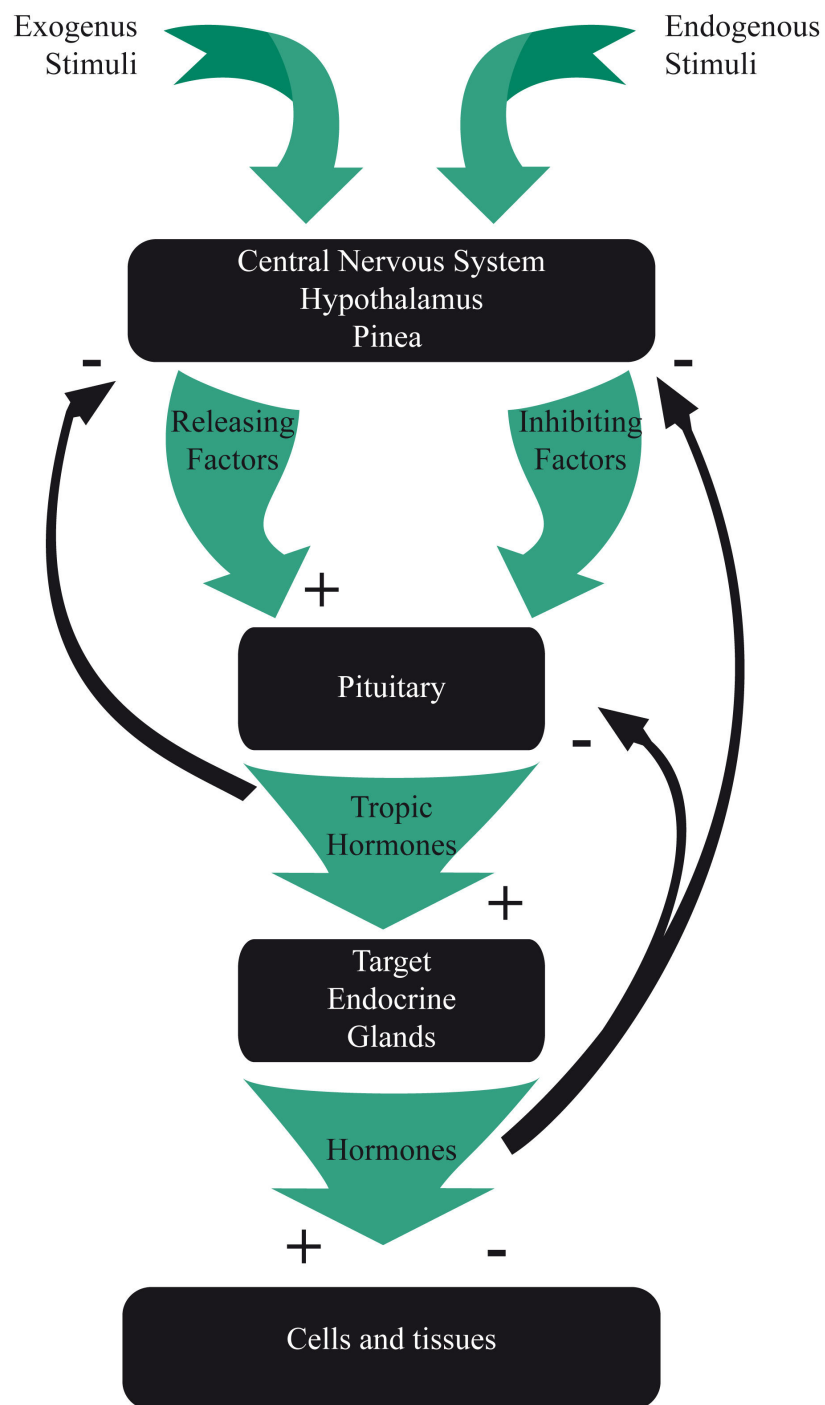


Figure 5: Simplified representation of stimulatory (+) and inhibitory (-) mechanisms among different part of the mammalian endocrine systems (figure by D. Scotti).

Reproduction in pinniped species is highly seasonal with the exception of monk seals which inhabit low latitude and Australian sea lions (Atkinson and Gilmarin, 1992, McKenzie et al., 2005). As in other mammals, gonadotropins released from the pituitary gland stimulate on one hand ovarian and testicular activities, resulting in ovulation and spermatogenesis, respectively, and on the other hand the associated behaviours of receptivity in females and rut in males (St. Aubin, 2001). In phocids and otariids, the duration of foetal development is shorter than the interval between breeding seasons. In order to overcome this temporal difference, phocids and otariids have adopted a delayed implantation of the blastocyst to allow parturition to occur at a time of year most suitable for successfully rearing pups (Atkinson, 1997). The day length is likely to be a critical factor for implantation, although the hormonal cascade that triggers implantation has not yet been documented.

Testosterone is the main androgen in male mammals and is mainly secreted by the testes. Testosterone concentrations in phocids and otariids increase for 1 to 3 months at the start of the breeding season (typically in spring), but decline to baseline levels after breeding ends. Testosterone is directly involved in spermatogenesis by differentiating the germ cells in the seminiferous tubules (Atkinson, 2002). In most male marine mammals, the seasonal testosterone pattern coincides with increased size of the testes and accessory reproductive glands. Increased size of the testes is the consequence of the increase of sperm volume in the seminiferous tubules and epididymes (Atkinson, 2002). Testosterone has also been associated with impairment of the immune function. The hormone interacts with the immune system at the level of both individual cells involved in the humoral- and cellular-mediated immunity and glands or tissues implicated in immune functions (reviewed in Grossman, 1985). Several studies show the consequences of high testosterone concentration on the individual health and that males with prolonged high testosterone concentrations may be more susceptible to disease or parasitism and ultimately, may reduce individual life span (Folstad and Karter, 1992, Moller and Saino, 1994, Zuk, 1996, Hoby et al., 2006).

1.7 PARASITES OF PINNIPEDS

Parasites have long been ignored in ecology because they are of small size and often

hidden from view. However, parasites are omnipresent and play important roles in ecosystems. Parasites increase their fitness by exploiting hosts for food, habitat, reproduction, and dispersal. Parasites reduce host fitness in many ways, ranging from general or specialized pathology (e.g. castration), impairment of secondary sex characteristics (e.g. elongated and ornamental-shaped birds' tails) , to the modification of host behaviour (e.g. infected hosts may be more susceptible to predation for transmission to other hosts) (Foreyt, 1997).

Parasite classification is based on a variety of aspects including parasite interactions with their hosts and parasite life cycles. Endoparasites of pinnipeds range from Protozoans to Helminths. The later tends to be the predominant group and have the greatest impact on health in pinnipeds (Dailey, 2001). Helminths, the trematodes, cestodes, nematodes, and acanthocephalans, infest several parts of the body, including the gastro-intestinal tract, respiratory and circulatory systems, liver, biliary system, pancreas, and connective tissues (Foreyt, 1997). Ectoparasites of pinnipeds range from mites to lice. The impact of these parasites on the host health depends upon the numbers infecting the host. Heavy loads of lice can cause irritation and alopecia. They are often secondary to debilitation and may have an indirect impact on diseases such as anaemia (Dailey, 2001). The major parasites of pinniped species are summarized in Table 2 and the pathogenic signs reported in pinnipeds are summarized in Table 3.

The life cycles of many parasites demand multiple hosts of different species and rely on predator-prey or other stable ecological interactions to get from one host to another in order to complete their life cycle (Foreyt, 1997). Although parasites are often left out from the field of food webs, they generally occupy the top position, functioning like keystone species, reducing the dominance of superior competitors, and allowing competing species to co-exist.

Table 2: Major parasites of Pinnipedia (seals, sea lions, and walruses) (Foreyt, 1997).

Trematodes <ul style="list-style-type: none"> ▪ Cryptocotyle ▪ Zalophotrema ▪ Pricetrema 	<u>Location</u> Intestine Liver Intestine
Cestodes <ul style="list-style-type: none"> ▪ Diphyllbothrium ▪ Phyllobothrium 	Intestine Tissue
Nematodes <ul style="list-style-type: none"> ▪ Anisakis ▪ Ascaris ▪ Contracaecum ▪ Dipetalonema ▪ Dirofilaria ▪ Ostostrongylus ▪ Parafilaroides ▪ Porrocaecum ▪ Terranova ▪ Uncinaria 	Stomach Intestine Stomach Heart Heart Lung Lung Stomach Stomach Intestine
Acanthocephala <ul style="list-style-type: none"> ▪ Corynosoma 	Intestine
Ectoparasites <ul style="list-style-type: none"> ▪ Antarctophthirus (louse) ▪ Echinophthirius (louse) ▪ Halarachne (nasa mite) ▪ Lepidophthirus (louse) 	Skin Skin Nasal sinuses Skin

Table 3: Summary of pathogenic parasites reported in pinnipeds

Parasites	Clinical signs reported in pinnipeds	Reference
Gastrointestinal tract <i>Uncinaria</i> (nematode)	Anaemia, peritonitis and haemorrhagic enteritis leading to bacteraemia and death in young northern fur seals, California and New Zealand sea lions	(Lyons et al., 2000, Castinel et al., 2006)
<i>Anisakis</i> , <i>Pseudoterranova</i> , <i>Phocascaris</i> (nematode)	Gastritis, gastric ulceration, enteritis, diarrhoea, dehydration, anaemia, and gastric perforation, and peritonitis in otariids and phocids	(Young and Lowe, 1969, Ridgway et al., 1975, Lauckner, 1985, Fletcher et al., 1998, Lehnert et al., 2007)
<i>Cryptocotyle</i> , <i>Rossicotrema</i> , <i>Phagicola</i> , <i>Stictodera</i> , <i>Phocitrema</i> , <i>Microphallus</i> , <i>Maritrema</i> , and <i>Ogmogaster</i> (trematode)	Colitis in elephant seals	(Dailey, 2001)
<i>Diphylllobothrium</i> (cestodes)	Pathogenic signs vary widely, but the effects are more likely to occur when large number obstruct the intestinal lumen	(Lauckner, 1985, Ionita et al., 2008)
Respiratory and circulatory system <i>Filaroides/Parafilaroides</i> (nematode)	Associated to pneumonia in stranded pinnipeds	(Lauckner, 1985)
<i>Otostrongylus circumlitus</i> (nematode)	Anorexia, depression, dehydration, neutrophilia, disseminated intravascular coagulation, and ultimately, death in elephant seals	(Gulland et al., 1997, Lehnert et al., 2007)
<i>Acanthocheilonema</i> (nematode)	Mainly pathogenic to young phocids; anorexia, dyspnea, coughing, and erratic breathing	(Lehnert et al., 2007)
Liver <i>Zalophotrema hepaticum</i> (trematode)	Aberrant migration in California sea lions	(Fauquier et al., 2004)

1.7.1 NZFS - parasites

Boren (2005) reports the common causes of death from post-mortems of 46 individuals collected from Ohau Point breeding colony located on the east coast of New Zealand. The determinant factors in cause of death were significantly different based on age class. Gastrointestinal parasites have been reported in all juveniles post-mortemed (n=10). Gastrointestinal parasites and hookworms have been reported in some individual pups aged 0-50 days. Lungworms have been reported in some individual pups, juveniles, and adults. Finally, flukes have been reported in individual pups aged 51 days-weaning (Boren, 2005). Boren (2005) examines the potential for intestinal parasites to affect pup growth in *A. forsteri* using anthelmintic treatment. She shows no difference in survivorship between the control and test group, from which it appears that hookworm has little effect in *A. forster* pups. In contrast *Phocarcos hookeri* (New Zealand sea lion) pups seem to be heavily affected, and hookworm accounted for 16.4% of juvenile mortality (Castinel et al., 2007). Genetic composition and diversity between these species might be an important factor in host resistance to infectious agents (Chapter 3).

1.8 BRIEF DISCUSSION OF THE PROJECT GOALS

The stability and increasing abundance of New Zealand fur seal populations provides the opportunity to use this study model for investigating polygynous mating systems and microevolutionary processes associated with life histories of dimorphic polygynous mammals.

Parentage analysis has enabled the examination of mating success, the identification of pedigrees, and the elucidation of social organisation, greatly enhancing our understanding of mating systems (Chapter 1). However, such analyses are not without pitfalls, with erroneous assignments common in open systems (i.e. when parental and offspring samplings are incomplete). We investigated the effects of the user-defined parameters on the accuracy of parental assignment in CERVUS, the most commonly used parental assignment programme, and PASOS, a relatively recent, but increasingly popular addition to the parentage assignment software (Chapter 2). In addition, we analysed the effects of the intrinsic bias in likelihood assignment approaches towards assigning higher

probability of parentage on individuals with rare alleles and those with heightened offspring-parent matches, which increase with the number of homozygous loci (Chapter 3).

In polygynous mammals, successful males mate with multiple females by competing with and limiting the access of other males to females. When the status of many males (age, size, health, genetic etc.) prevents them from achieving the primary mating tactic, theory predicts selection for a diversification of male mating tactics. Recent studies in pinnipeds have shown that observed male mating success was correlated to male paternity success in some species (elephant-seals), but not in others (grey seals). The existence of alternative mating strategies can explain those discrepancies. Chapter 4 focuses on the polygynous New Zealand fur seal *Arctocephalus forsteri*, predicting that 1) competition for females is likely to cause a diversification of male mating tactics; and 2) that alternative tactics can yield reproductive success. Our results indicated three male behavioural profiles; one corresponded to large territorial males and two illustrated a continuum of alternative tactics employed by non-territorial subordinate males. In addition, we estimated the reproductive success of male mating tactics and highlight that holding a territory is not a necessary condition for reproductive success in a population of otariids.

The degree of sexual size dimorphism in polygynous species is expected to increase with the degree of intra-sexual competition and in turn with the degree of polygyny. The life history of an individual is the pattern of resource allocation to growth, maintenance, and reproduction throughout its lifetime. Both females and males incur viability costs of mating and reproduction. However, male viability costs due to increase growth and male-male competition can be greater than female viability costs of mate choice and reproduction. Although an abundant literature on sexual dimorphism in morphology, physiology, and parasite infections is available, little is known on the intra-sexual differences in physiology and parasite infections associated to the reproductive success of individuals employing different mating strategies. Chapter 5 examines the viability costs of sexual selection between territorial and subordinate males New Zealand fur seal and uses the data on reproductive success in Chapter 4 to allow a multivariate analysis.

Last, in order to complete the above study, I have successfully developed and applied a number of new technical methods that are not presented in the main thesis. These include the use of simplified and cost-effective conductive media for a rapid and high resolution of small DNA fragment using polyacrylamide-based electrophoresis (Negro et al., 2006), and new genotyping protocols using hair follicles as a source of DNA (Caudron et al., 2007) (Appendix). I also gather the genetic data and performed the genetic data analysis, and wrote part of a paper describing the rearing of two putative twin *A. forsteri* pups to weaning (Dowell et al., 2008) (Appendix). Finally, while tangential to this study, I contributed genotypic data for NZFS that was used for an analysis of hybridisation among three sympatric species of fur seal at Macquarie Island following historical population extinction (Lancaster et al., 2006).

Chapter Two

Paternity testing in open systems and an evaluation of the effects of user-defined parameters on two parental allocation programs, CERVUS and PASOS, using simulated genotypes

Negro S.S.¹, Naduvilezhath L.², and Gemmell N.J.^{1,3}

¹Molecular Ecology Laboratory, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

²Institut für Informatik, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

³Centre for Reproduction and Genomics, University of Otago, PO Box 913, Dunedin, New Zealand

Submitted manuscript, please do not quote without first contacting the authors

Abstract

Genetic data coupled with increasingly sophisticated computational tools enable the examination of fine scale relationships within and between populations, including the determination of parentage. Parentage analysis has enabled the examination of mating success and the identification of pedigrees and social organisation, greatly enhancing our understanding of mating systems. However, such analyses, which are more often than not based on likelihood statistics, are not without their pitfalls with erroneous assignments common in open systems in which a significant proportion of parents are unsampled. In this paper firstly we investigated how varying the user-defined parameters in CERVUS v3.0 and PASOS v1 affected the accuracy of parental assignment, secondly we investigated the potential for combining the results of the two parental assignment programmes to raise the probability of correct parental allocation. We explored these questions using simulated genetic data derived from data sourced from an empirical study of the New Zealand fur seal. Our study shows that inaccurate user-defined parameters in CERVUS and PASOS can lead to highly biased output e.g. the assignment rate at 95% CL of offspring with a sampled known mother to sampled males decreased from 58% to 32% when the proportion of candidate males sampled in the parameter options decreasing 4-fold. We found that the use of both CERVUS and PASOS for parentage assignment can increase the likelihood of correctly allocating offspring to sampled parents to 97% in our study system. Incorrect parental assignment can bias estimates of various biological parameters, such as lifetime reproductive success and mate choice preference, and hence bias ecological and evolutionary interpretations. Here, we propose solutions to increase the power of parentage assignment and hence decrease the bias in biological parameter estimates. We find that the microsatellite genotyping error rate and the number of candidate males need to be estimated with precision in order to achieve accurate parentage assignments. To minimize erroneous assignment decisions, the number of male-offspring pair and/or male-mother-offspring triad mismatches should be taken into account as well as the number of offspring-parent matching alleles and rare alleles.

2 Paternity testing in open systems and an evaluation of the effects of user-defined parameters on two parental allocation programmes, CERVUS and PASOS, using simulated genotypes

2.1 INTRODUCTION

High-throughput genotyping techniques provide an unprecedented amount of genetic data which when coupled with increasingly sophisticated computer programs enable fine scale relationships within and between populations to be evaluated. When applied within populations such data and analyses often provide great insights into mating systems through improved understanding of parentage (Westneat and Sherman, 1997, Ambs et al., 1999, Gemmell et al., 2001, Hoffman et al., 2003), social organization (Conrad et al., 1998), and sexual selection (Payne, 1979), all of which are important for a detailed understanding of population dynamics, evolution, and conservation.

Parental assignments using genetic data are conceptually simple, but require a degree of computation to achieve matches. Such matches are seldom made with certainty, but instead represent probabilities or likelihoods, that parental allocations are correct. System type (closed or open) is the primary factor that determines which technique is most appropriate to use (Jones and Ardren, 2003). In closed systems (where all offspring and candidate parents are sampled), the probability of achieving complete parental assignment is high (Oka and Takenaka, 2001). As the proportion of candidates that are sampled drops (open systems), the number of true parents absent from the parental genotype files increases and the likelihood of success in parental analyses decreases (Neff et al., 2000a, Neff et al., 2000b). Correct parental assignment to specific parental genotypes becomes impossible for some offspring and some offspring may be assigned incorrectly. As the population sample becomes less favourable for such studies, a greater number of markers with higher polymorphic information content are required for a successful study. However, genetic data from open systems may still be useful for evaluating mating system parameters (Ritland, 2002).

Two types of erroneous assignments can arise during parentage analysis: 1) failure to assign the correct parent when it is present in the sample and 2) assigning the offspring to a false parent, which may occur whether the true parent is absent or present. Several factors influence the assessment of confidence in assignments and the parental assignments by programmes using categorical allocation. The major cause of mismatches between offspring and their biological parents is through microsatellite genotyping errors (Hoffman and Amos, 2005). Mutation, null alleles, and the use of limited number of loci can also result in incorrect assignment (Bernatchez and Duchesne, 2000, Neff et al., 2000b, Hoffman and Amos, 2005). The proportion of candidate parents sampled from the population is another important constraint on the accuracy of parentage studies. Partial sampling leads to two major problems. First, assignment techniques require knowledge of the total number of candidate parents in the population because this value is used in, and is sensitive to, the assessment of confidence in assignments (Nielsen et al., 2001). Second, if the programmes that assign parent pairs or use a parent-pair assignment algorithm fail to sample either member of a breeding pair, correct assignment becomes impossible. In addition, the presence of family structure in the studied population can be problematic when some of the candidate parents are related to each other. However, a strict exclusion approach and an increase of the number of loci (if necessary) may still give certainty. Finally, a recent study investigating intrinsic biases in likelihood assignment approaches shows that individuals with rare alleles and those with increased offspring-parent matches (which increase with the number of homozygous loci) have higher assignment probability of parentage (Negro et al., In Review).

A few studies have previously examined the efficiency of parental assignments for parentage programmes using a retrospective assessment (e.g. Slate et al., 2000), while others have used two parental allocation programmes simultaneously on the same data set to evaluate the relative performance of these assignment programmes (e.g. Newpat and CERVUS; Worthington Wilmer et al., 1999, Cerchio et al., 2005, Hoffman et al., 2003). However, none of these studies has investigated the effects of the user-defined parameters on the parental assignments, or combined the results of multiple parental allocation programmes to raise the probability of correct parental allocation. In this paper, we used simulated genetic data to analyse the effects of the user-defined parameters (i.e. the number of candidate males, the proportion of candidate males sampled, the genotypic error rate, and the presence of relatives

in the sample) on paternity assignment in CERVUS v3.0, the most commonly used parental allocation programme in molecular ecology (Marshall et al., 1998, Kalinowski et al., 2007), and PASOS a relatively recent, but increasingly popular addition to the parentage assignment software (Duchesne et al., 2005). We showed that the use of inaccurate user-defined parameters results in biased parental assignment and propose solutions to decrease the bias in biological parameter estimates and increase the power of parentage assignment. We also used these programmes conjointly to demonstrate an increase in the likelihood of correct offspring assignment to the sampled parents.

2.1.1 *Parental allocation programme*

An exclusion-based approach (Chakraborty *et al.*, 1974) is a useful starting point for parentage analyses, but it is generally not sufficient for paternity inference when multiple males are genetically compatible with each of the offspring tested, a common situation in populations that are large and have a propensity to philopatry or that have low polymorphism. In such instances, likelihood-based approaches that assign paternity to the most likely male if several males are not excluded are preferred (Meagher, 1986). Here, we evaluate two programs that utilise a likelihood framework, CERVUS and PASOS, in part because they allow for genotyping errors, missing data, and the effects of incomplete sampling in open systems. In addition, both programs have a range of unique features that are intended to minimize the effects of the field, biological and laboratory errors and other complications that often affect studies of parentage.

CERVUS works on a likelihood-based approach that assigns paternity to the most-likely male with a known level of statistical confidence (probability that an assignment is correct). It has been used for parental analysis on a wide range of organisms, including insects, fish, birds, and mammals, in both closed and open systems. CERVUS v3.0 can perform calculations of parental pair likelihoods (parental pair analysis) in addition to individual male or female likelihoods (paternity and maternity analysis).

The likelihood-based approaches implemented in CERVUS involve a simulation and a paternity module, which require that allele frequencies be first calculated by CERVUS from the source data set. Simulation parameters in CERVUS take into account the number of

candidate males, the proportion of candidate males sampled, missing genotypes, and genotyping errors, while no parameters have to be defined in the paternity analysis module. However, the paternity analysis module uses the parameter values set in the simulation module while running the paternity analysis. The simulation for paternity testing works in three steps. First, the CERVUS simulation calculates a log-likelihood ratio or LOD score (the likelihood of paternity of a particular male relative to the likelihood of paternity of an arbitrary male) for each candidate male. Exclusions do not occur (providing the scoring error rate is greater than zero) because the program allows for scoring errors, hence candidate males with several mismatching loci are given a very low or negative LOD score. Second, it ignores all candidate males whose log-likelihood ratio is zero or negative, finds the two most-likely candidate males, and calculates delta, the difference in their LOD scores (Δ , the logarithm of the ratio of likelihood ratios). Third, the last stage of the simulation is to compare the distribution of Δ scores for cases where the most-likely male was the true father (N_I) with the distribution for cases where the most-likely male was not the true father (N_f) (Figure 1). Following this comparison, the critical values of Δ can be identified (Table 2). The critical value of Δ is the criterion that the most-likely males have to fulfil to be assigned paternity at a defined confidence level (CL, 80% or 95%). The most-likely males with Δ scores that are lower than the critical value of Δ will not be assigned any paternity for a defined CL.

PASOS was originally designed for parentage research in open systems where both parents are unknown (Duchesne et al., 2005). The user has to know the proportion of missing parents to have accurate assignments and to estimate CLs and correctness, the probability that assignments are correct (Nielsen et al., 2001, Duchesne et al., 2005). At the same time, the user has to allocate offspring correctly to obtain such estimates. To escape from this circularity, PASOS provides a reliable estimate of the proportion of missing parents by assigning offspring to the general category of the uncollected (UC) in addition to the collected parents.

PASOS combines an approach based on parental pair likelihoods with a subsequent filtering procedure which tolerates errors up to some specified number of offsets (the Maximum Offset of Tolerance, MOT; maximum allelic distance measured in microsatellite repeat units from the focal parental allele). The allocation process starts with a search for the most likely

pair among all potential pairs of sampled parents. Simulation parameters in PASOS take into account the MOT and genotyping errors while only the MOT parameter has to be defined in the parental allocation module (the same MOT value is used in the parental analysis module and the simulation module).

The features from PASOS and CERVUS are detailed in Table 1. PASOS differs from CERVUS in essentially two ways. First, PASOS provides an estimate of the proportion of uncollected parents (UC) directly computed from specific parental and offspring genotypes. Hence, PASOS produces two kinds of assignments: assignment to a known collected parent and assignment to a category of uncollected parents, *i.e.* it positively states that the parent is not listed (CERVUS makes only allocation to the sampled parents). Second, the simulations in PASOS then use estimates of the proportion of missing parents to output correctness estimates (the simulations in CERVUS utilise user-define estimates of the proportion of candidate males sampled to output critical values of Δ to be used as criteria for assigning males with defined confidence intervals in the paternity analysis). Once obtained, the PASOS simulated offspring allocations should be compared with the proper offspring allocations. A reasonable fit of the simulated and proper offspring allocation curves should be considered for processing to the next steps of the parental assignment analysis.

Table 1: Comparison of the features of two computer programmes, CERVUS and PASOS, for parental inference in natural populations.

	CERVUS	PASOS
Type of genetic marker	Diploid codominant	Diploid codominant
Input files	Genotypes of offspring and sexed or unsexed parents	Genotypes of offspring and sexed or unsexed parents
Missing genotype	Allowed	Not allowed in parental files
Parents of each offspring known <i>a priori</i>	Neither or one	Neither or one
Type of assignment	Paternity/maternity/parental pair	Parental pair
Assignment	To collected (sampled) parent	To collected parent and to uncollected parent
Method of parentage analysis	Categorical likelihood*/extended version of exclusion that takes into account genotyping errors	Categorical likelihood*/extended version of exclusion that takes into account genotyping errors and tolerates errors up to some number of offsets, maximum offset of tolerance** (MOT)
Available functions:		
HW test	Yes	No
Null allele frequencies estimate	Yes	No
Relatives among the candidate parents	Yes	No
Statistical confidence levels	80% and 95%	Overall assignment correctness rates
Identical genotypes screening	Yes	No
Exclusion probabilities calculation	Yes	Gives minimum subset of loci
Assignment filtering	No	MOT
Estimated proportion of uncollected parents	No	Yes
Ability to accomodate "error"***:		
Null Alleles	Yes (partially)	Yes
Genotyping error	Yes	Yes
Mutation	Yes	Yes

* The categorical technique uses likelihood-based approaches to select the most likely parent from a pool of non-excluded parents. ** MOT: the maximum allelic distance measured in repeat units from the focal parental allele. *** Jones and Ardren (2003).

2.2 METHODS

2.2.1 *Simulated Genetic data*

For this study, we created a simulated data set that exhibited the common limitations observed in real field data. This simulated data (input file) was used in all CERVUS and PASOS analyses to be able to achieve a detailed examination of assignments and misassignments under a variety of user defined programme settings. A Java programme (available on request) was used to generate the input file containing simulated- male ($n = 35$), female ($n = 100$) and offspring ($n = 100$) genotypes using the allele frequencies observed in a wild New Zealand fur seal population that has been the focus of paternity studies since 2003 (Caudron et al., In prepa.) (Table 2). Simulated-offspring were generated by randomly mating parents. Each parent/offspring relationship was recorded in a spreadsheet file to determine the accuracy of the parental programme assignment. Furthermore, because sampling constraints routinely prevent researchers from working in a closed system this limitation was simulated by randomly removing some females (50%, $n = 50$) and males (29%, $n = 10$) from the output files which corresponded to the New Zealand fur seal data in Caudron et al. (In prepa.). Likewise, because males rarely have equal potential for dominance and access to females, with polygyny displayed by over 90% of mammals (Clutton-Brock, 1989), 10 out of 25 simulated-males left in the output file were not assigned any pups, which simulated the variation in male reproductive success due to intra-specific competition in polygynous systems observed in Caudron et al. (In prepa.). The range of reproductive success among simulated males ranged from zero to seven pups, with 57 out of 100 offspring allocations distributed among the 25 candidate males retained.

Genotyping errors are common and increase with the number of microsatellite scored (Jones and Ardren, 2003, Hoffman and Amos, 2005). Errors occur at a rate of 0.5 to 3% when scoring loci and the probability of scoring error decreases with the increase of the allelic distance from the focal parental allele (Duchesne and Bernatchez, 2007). In order to incorporate errors in our simulated genotypes that follow the above error probability distribution encountered in real genotype files, a restricted error model was included in our Java program. The distance structure of alleles as a function of allelic distance measured in microsatellite repeat units from the focal parental allele X was as follows: $X-2$ offsets, $X-1$ offset, X , $X+1$ offset, $X+2$ offsets, where one offset is defined as the smallest allelic distance

between any two alleles scored in the locus. The distribution for a 2% error rate was 0.002, 0.008, 0.98, 0.008, and 0.002.

The two steps most commonly used for genetic analysis are the examination of the basic properties of the data (e.g. Hardy-Weinberg equilibrium, HWE, and linkage disequilibrium, LD, measurement), followed by more specialized analyses (e.g. parentage analysis) (Excoffier and Heckel, 2006). We checked the simulated genetic data for HWE and LD and used these genotypes to evaluate the effects of the user-defined parameters.

2.2.2 *User-defined parameters and paternity assignment*

CERVUS is widely used for research on mammalian reproductive ecology, with more than 1300 cites to date on the Web of Science. Mother-offspring pairs are often known in mammalian research because maternal care is part of the reproductive success. Researchers usually sample both offspring and mother while maternal care takes place since females generally nurse their own offspring. In some cases, researchers sample the offspring without the mother for logistics reasons. Considering the number of individuals in the candidate male (CM) and candidate female (CF) pools for a single offspring assignment, the sample scenario used for evaluating the effects of the user-defined parameters and the accuracy of paternity assignment in CERVUS was: CF = 1 (known mother sampled, $n = 50$) and 0 (mother unsampled, $n = 50$) and CM > 1. Analysis of maternity (CF > 1 and CM = 1 or 0) has the same implications. The assignment rule used in CERVUS reported offspring-male pair or offspring-mother-male triad (if the known mother is sampled) on defined confidence levels (80% or 95% indicated by + or * in the parental analysis file respectively). The offspring unassigned to a father include cases with undefined CL. The most-likely father (indicated by -), males in the list of positive LOD, and males in the list of negative LOD have undefined CL.

The sample scenario used for combining the paternity results from CERVUS and PASOS was: CF = 1 and CMs > 1. For a valid comparison with CERVUS, although it is a laborious task, we informed PASOS that the mother of a particular offspring is known *a priori* by running an allocation for this particular offspring with a parental file containing a single genotype for the known mother. The allocation rule used in PASOS reported the most-likely

parental pair (with a defined correctness rate) among all potential pairs of collected and uncollected parents. PASOS can provide an estimate of the number of candidate parents because it provides a reliable estimate of the proportion of unsampled parents in addition to the sampled parents. We examined the accuracy of the PASOS estimates of the proportion of CMs unsampled and CMs sampled (i.e. total number of CMs).

The user-defined parameters of CERVUS and PASOS are designed to minimize the problems encountered when complete sampling is impossible. In order to estimate the sensitivity of CERVUS to the user-defined parameters, we used different parameter values based on simulated genotypes from the Java program. Three types of paternity inference tests were run in CERVUS simulations (which are used in the paternity analysis module) with different parameter values: (1) sets with 0% loci mistyped (using input files with no scoring errors); (2) sets with 2% loci mistyped (using input files with 2% error rate); and (3) sets with 2% error rates considering relatives among candidate males. For each of the three types of tests, different proportions of candidate males sampled were tested (i.e. 1 (25 CMs), 0.714 (35 CMs), 0.555 (45 CMs), 0.416 (60 CMs), 0.25 (100 CMs)) to evaluate the sensitivity of the program to the estimate of the proportion of candidate parents sampled from the standpoints of the accuracy of the assignments and the defined CLs. The relatedness parameter (advanced parameter) in CERVUS is an on/off switch specifying (i) the number of relatives among the candidate males sampled that are related to the candidate males or the mothers or the offspring, and (ii) the relatedness estimation. The relatedness estimator, R , for our data (Queller and Goodnight, 1989) was calculated using the program GenAlEx v6 (Peakall and Smouse, 2006). Among the candidate males sampled, 10 were related with a mean R of 0.401. The parameters used in PASOS simulations were: 2% loci mistyped (input files with 2% error rate) and varying values of MOT. The MOT parameter was calibrated before parental allocation analysis by allocating the offspring from known mother-offspring pairs with MOT = 1, 2, 3, and 4 until all (or nearly all) mothers from the known pairs had been allocated. The smallest MOT value that allowed allocation of nearly all mothers from known mother-offspring pairs was retained. Higher MOT values would have resulted in a loss of power, but too small a MOT value necessarily results in over-exclusion (elimination of true parents). Finally, various rates of scoring error (1%, 2%, 3%, and 5%) were used in CERVUS and PASOS simulations to analyse the confidence levels of parental assignment

outputs. The other parameters were set at defined values (the closest to the characteristics of the input files) and kept unchanged: CMs = 35 and proportion of CMs sampled = 0.714.

Paternity analysis from CERVUS and PASOS was combined to gauge whether using multiple programs raised the probability of correctly assigning parentage. We recorded the common true and false father assignments, the common true and false unassigned offspring (in CERVUS) and assignments to uncollected parents (in PASOS), and calculated the common assignment correctness i.e. $1/2[\text{common true father assignments}/\text{common true and false father assignments} + \text{common true unassigned offspring and UC assignments}/\text{common true and false unassigned offspring and UC assignments}]$.

2.3 RESULTS

2.3.1 User-defined parameters

Prior to any analyses, we checked all microsatellites used in this study for their appropriateness as population genetic markers by analysing different statistical outputs (e.g. Hardy-Weinberg equilibrium) using CERVUS v3.0 (Table 2). For each of the three types of paternity inference tests in CERVUS (0% error rate; 2% error rate; 2% error rate with relatives among sampled candidate males), different numbers of candidate males (25, 35, 45, 60, 100) were tested. Figure 1 shows the distributions of Δ scores (the difference in the LOD scores of the two most-likely candidate males). Depending on the ratio of the number of true fathers (N_t , black bars) to false fathers (N_f , grey bars), pairs of distributions of Δ for the true fathers and false fathers fall into three categories:

1. $N_t > N_f$. The critical value is low and success rate is high.
2. $N_t < N_f$. The critical value is high and the success rate is low.
3. $N_t \approx N_f$. The critical value is intermediate.

The critical Δ scores and the success rate (proportion of males including true and false fathers fulfilling the required criterion) of paternity tests and simulation tests output by CERVUS are summarized in Table 3. In all three types of paternity tests the success rate decreased as the number of candidate males in the parameter option increased.

The calibration of the maximum offset of tolerance (MOT) was achieved by undertaking parental allocation on a data set consisting of offspring from known mother-offspring pairs with MOT = 1, 2, 3 and 4 (Figure 2). The proportion of offspring allocated using MOT = 1 missed a proportion of mother-offspring pair allocations and over-exclusion occurred. The test with MOT = 2 is the smallest value showing that almost all mothers from the known pairs have been allocated. This MOT also shows the best fit between the proper and the simulated offspring allocation to the mother (Figure 2). This parameter value was thus retained for the paternity assignment analyses and comparison with CERVUS.

The estimate of the proportion of CMs sampled was 0.81 for PASOS, which is 10% greater than, but close to the real (true) CM sampled (0.71; 25CMs sampled out of 35 CMs) for our simulated data.

We examined the effects of increasing the error rate (1%, 2%, 3%, and 5%) on the critical values of delta and the proportion of simulated assignments at defined confidence intervals, 95% and 80% (Figure 3). Increasing the error rate in the parameter options increased the critical value of delta and decreased the simulated assignment rate.

2.3.2 Paternity assignment

The Java program generated simulated offspring and recorded information on each simulated parent for each offspring. The parental input files contained 25 out of 35 CMs. Fifty seven out of 100 offspring allocations were distributed among 25 CMs retained. We chose the parameters for the paternity analysis using CERVUS that matched the known values from our input files (CMs = 35, proportion of CMs sampled = 0.714, 2% error rate, relatedness on or off).

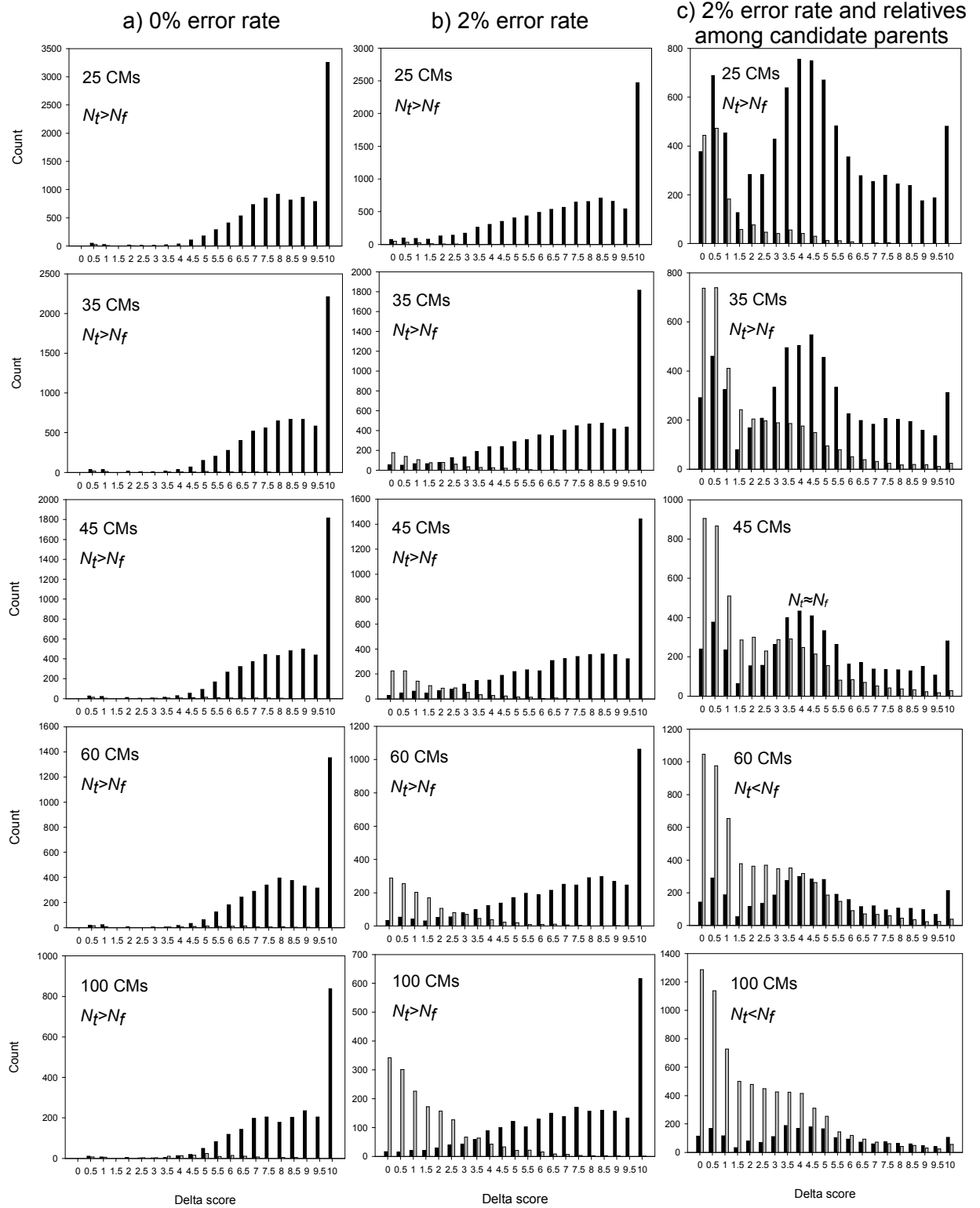
The paternity analysis by CERVUS and PASOS is summarized in Table 4. In CERVUS, the total numbers of assignments and the numbers of true and false father assignments were higher when the relatedness parameter was switched off. The numbers of true and false unassigned offspring were lower when the relatedness parameter was switched off. The false father assignment rates were higher when the mothers are unsampled than when the know

mothers are sampled. The common true father assignment rate (65%) is a measure that underestimated the true father assignment rate. The common assignment correctness was higher when combining CERVUS with the relatedness parameter switched on and PASOS assignments (97%) than when combining CERVUS with the relatedness parameter switched off and PASOS assignments (91% at 80% CL and 95% at 95% CL).

Table 2: Genetic diversity of the simulated genotypes at eight microsatellite loci using the allele frequencies of the New Zealand fur seal *A. forsteri*. A_N : allele number; He_O & He_E : observed & expected heterozygosity; PIC: polymorphic information content; HW: Hardy-Weinberg disequilibrium (S: significant, i.e. $p < 0.05$ after sequential Bonferroni correction for multiple tests, NS: non significant). NE-1P: non-exclusion probability when only one parent is known; NE-2P: probability of non-excluding two putative parents; NE-PP: non-exclusion probability of parent pair; NE-I: non-exclusion probability of identity; NE-SI: non-exclusion probability of sib identity. F(Null): null allele frequency.

<i>Locus</i>	A_N	He_O	He_E	PIC	NE-1P	NE-2P	NE- PP	NE-I	NE-SI	HW	F(Null)
HI16	12	0.840	0.818	0.790	0.543	0.368	0.190	0.060	0.357	NS	-0.0147
Lc5	8	0.783	0.771	0.731	0.629	0.450	0.269	0.091	0.389	NS	-0.0098
Hg4.2	20	0.851	0.849	0.836	0.442	0.281	0.104	0.034	0.335	NS	-0.0016
Pv11	9	0.697	0.719	0.688	0.669	0.485	0.281	0.109	0.419	NS	+0.0167
M11a	17	0.806	0.801	0.775	0.553	0.378	0.190	0.065	0.367	NS	-0.0032
Hg6.3	9	0.749	0.769	0.731	0.622	0.445	0.259	0.090	0.389	NS	+0.0124
Pv9	10	0.863	0.844	0.824	0.477	0.310	0.137	0.043	0.340	NS	-0.0144
3E3	6	0.594	0.576	0.523	0.825	0.668	0.500	0.233	0.521	NS	-0.0216

(i) Sampled known mothers (n = 50)



(ii) Unsourced mothers (n = 50)

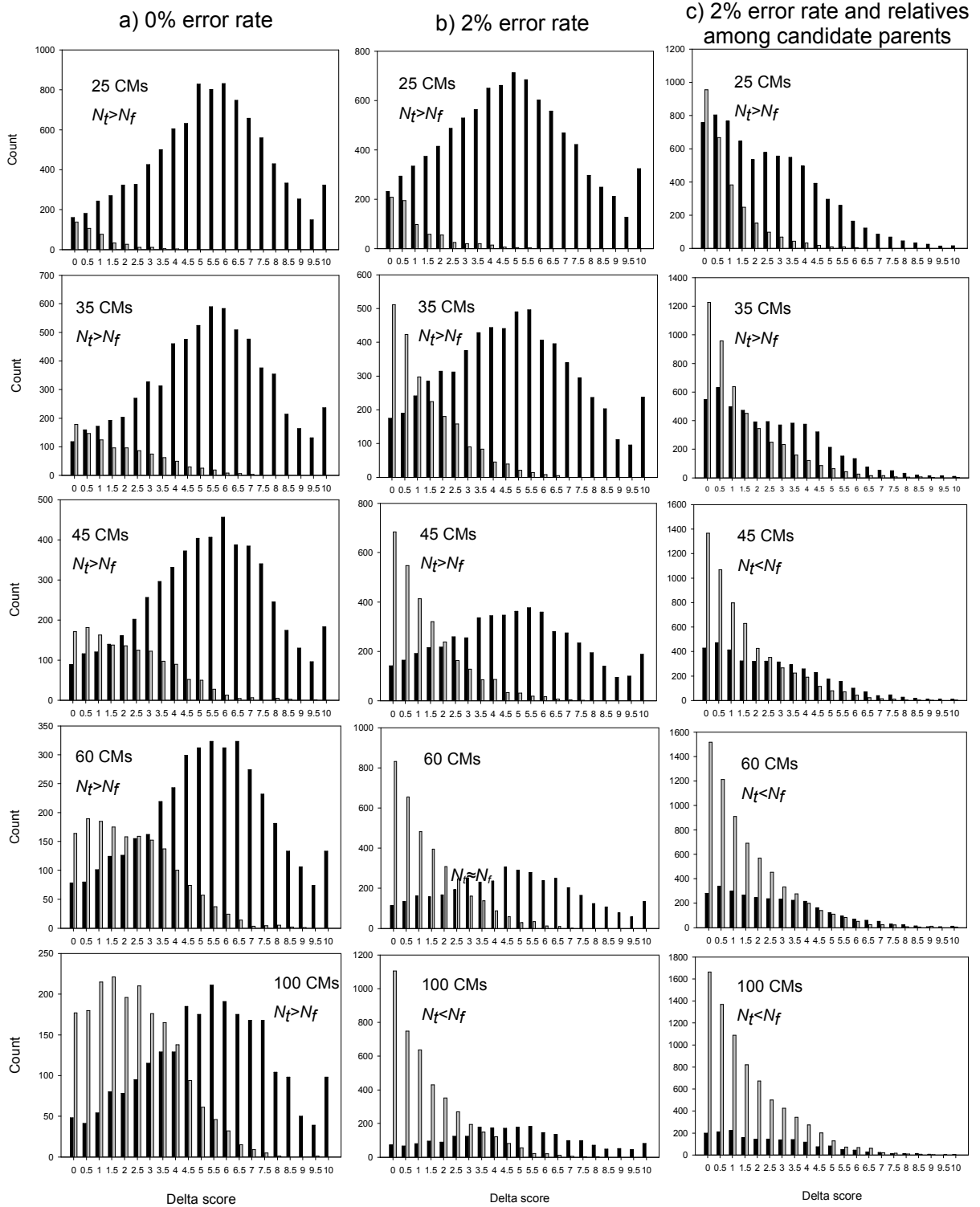


Figure 1: Distribution of delta (Δ) scores generated by CERVUS-simulations of paternity inference using allele frequencies from a New Zealand fur seal population. The parameter values were: conservative and relaxed error rate (0% and 2% respectively) and 10 relatives ($R = 0.401$) among the candidate males for 25, 35, 45, 60, 100 candidate males (CMs). Two types of simulations are shown: (i) when the mother was sampled and (ii) when the mother was not sampled. The histogram of N_t cases where the most-likely male is the true father (black bars) is interleaved with the histogram of N_f cases where the most-likely male is a false father (grey bars). Critical values of Δ are calculated from the degree of overlap of the two distributions. Table 2 shows the critical values of Δ for 80% and 95% confidence levels. Delta categories are labelled with their upper limits.

Table 3: The critical delta (Δ) scores and the success rate (proportion of males fulfilling the required criterion) of paternity tests and simulation tests (n=10 000) (in brackets) output by CERVUS. Simultaneous tests were carried out for paternity inference with sampled and unsampled mothers. Strict (95% confidence) and relaxed (80% confidence) criteria are shown. Different values for each user-defined parameter were set as follows: 25, 35, 45, 60, 100 candidate males (CM); strict and relaxed error rates (0%, 2%; input files with 0% and 2% error rate respectively), relatedness (R) parameter activated (+) or not (-) (10 CM sampled with a mean of 0.401 R value).

# CM	Error rate (%)	R	Sampled known mother (n=50)		Unsampled mother (n=50)	
			95%	80%	95%	80%
25	0	-	0*	0*	0*	0*
			68% (100%)	68% (100%)	72% (100%)	72% (100%)
35	0	-	0*	0*	2.86	0*
			68% (72%)	68% (72%)	50% (61%)	72% (79%)
45	0	-	0*	0*	4.27	0.23
			68% (56%)	68% (56%)	44% (39%)	72% (66%)
60	0	-	0*	0*	5.25	2.09
			68% (42%)	68% (42%)	28% (24%)	56% (45%)
100	0	-	0.75	0*	6.13	3.80
			68% (26%)	68% (26%)	16% (11%)	50% (23%)
25	2	-	0*	0*	0.57	0*
			58% (99%)	58% (99%)	64% (94%)	72% (99%)
35	2	-	1.45	0*	3.27	0.51
			46% (72%)	58% (77%)	44% (51%)	64% (79%)
45	2	-	2.57	0*	4.19	1.48
			40% (54%)	58% (65%)	36% (33%)	56% (57%)
60	2	-	3.27	0.56	4.85	2.48
			38% (40%)	50% (51%)	24% (21%)	44% (39%)
100	2	-	4.64	1.94	5.82	3.73
			32% (22%)	46% (30%)	16% (9%)	40% (20%)
25	2	+	1.63	0*	3.24	0.62
			46% (72%)	58% (99%)	44% (30%)	62% (78%)
35	2	+	14.25	2.95	10.50	4.33
			0% (0%)	38% (56%)	0% (0%)	34% (15%)
45	2	+	15.75	6.39	10.75	7.13
			0% (0%)	30% (16%)	0% (0%)	12% (2%)
60	2	+	14.88	8.52	999.00	999.00
			0% (0%)	22% (6%)	0% (0%)	0% (0%)
100	2	+	14.50	14.50	10.00	9.75
			0% (0%)	0% (0%)	0% (0%)	0% (0%)

*Critical value sets at 0 when $N_t/(N_t + N_f) \geq$ confidence interval

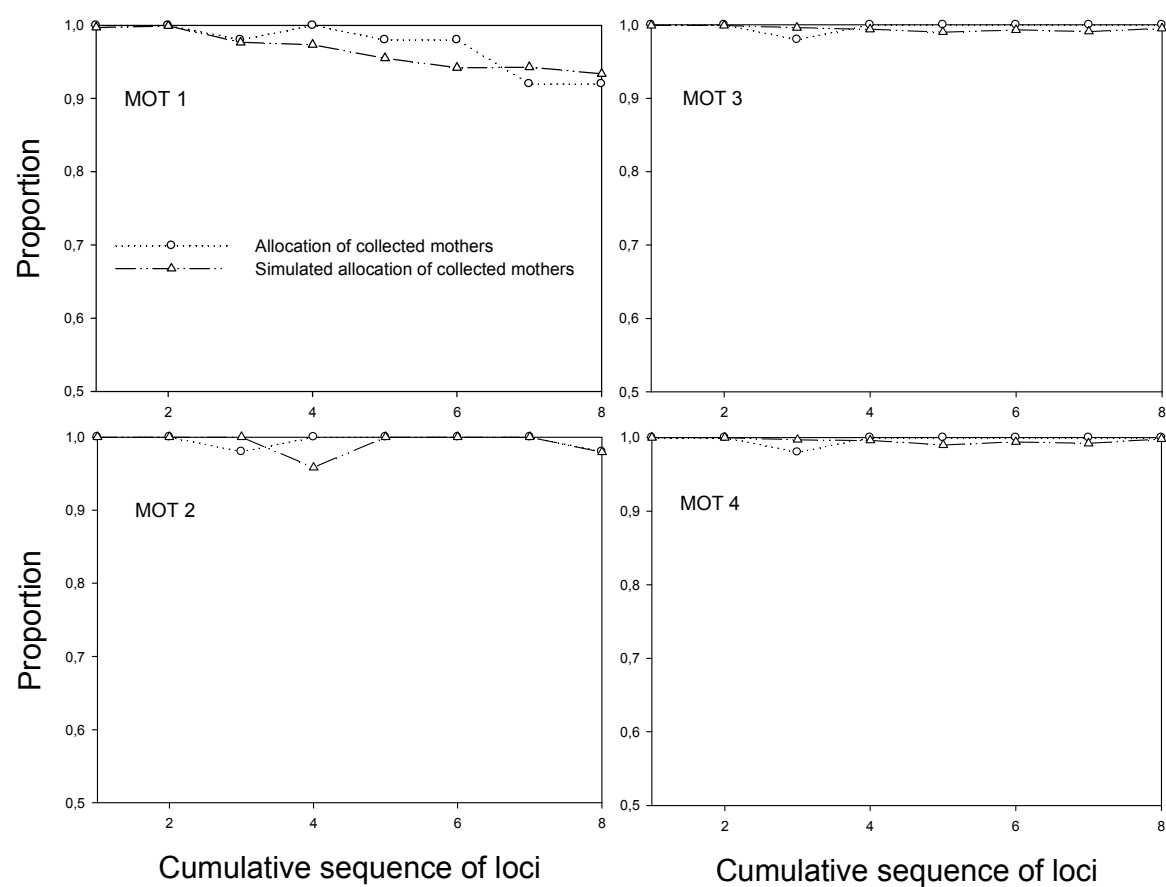


Figure 2: Calibration of PASOS maximum offset of tolerance (MOT; maximum allelic distance measured in repeat units from the focal parental allele) using mother/pup pair genotypes. The real allocation and simulated allocations of collected females are plotted using different values of MOT (1-4). The smallest MOT showing that nearly all mothers from known pairs have been allocated and showing a reasonable fit between the true and simulated allocation of collected females should be retained (here, MOT = 2).

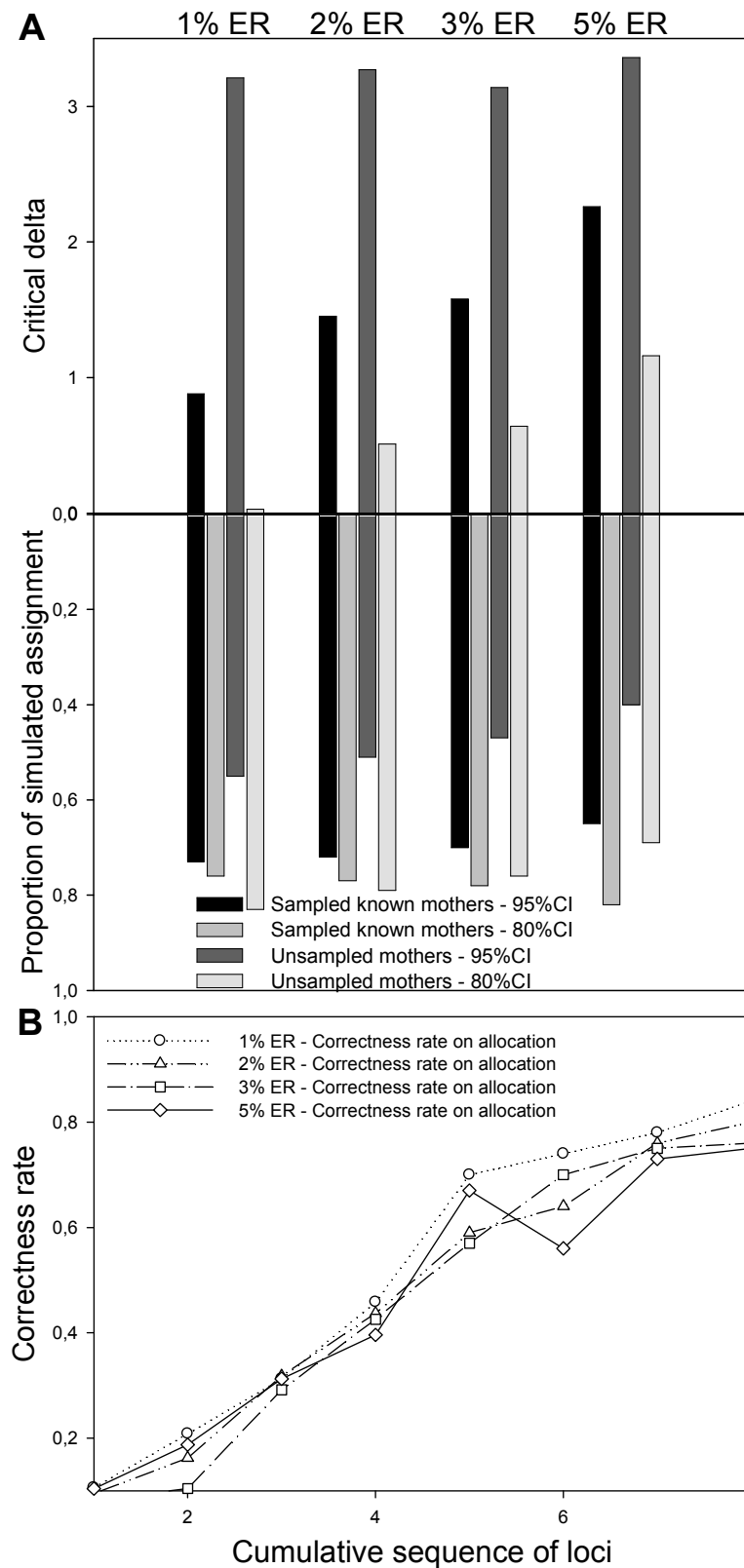


Figure 3: Different values of error rate (ER) and their effects on the critical values of delta (Δ) and the proportion of simulated assignments at defined confidence levels (95% CL, 80% CL) in CERVUS (A) and on the correctness rate in PASOS (B). The other parameters were set at defined values and kept unchanged.

Table 4: The total numbers of paternity inferences by CERVUS are shown along with the true/false assignments and true/false unassigned offspring. CERVUS parameters (i.e. number of candidate males, CM; error rate) were set according the input file characteristics. The effects of an advanced parameter (i.e. relatedness among CM sampled) on the true and false father assignments are also shown.

	Known mother sampled			Mother unsampled		
	CERVUS	PASOS	CERVUS	CERVUS	CERVUS	CERVUS
	35CM - 2% error - CM Relatedness	MOT = 2	35CM - 2% error 80% CL	35CM - 2% error - CM Relatedness	35CM - 2% error - 80% CL	35CM - 2% error 80% CL
Total assignment	19/50 (38%)	41/50 (82%)	29/50 (58%)	17/50 (34%)	32/50 (64%)	
True father assignment	19/29 (66%)	28/29 (97%)	20/29 (69%)	16/28 (57%)	21/28 (75%)	
True father as most-likely father (undefined CL*)	1/29 (3%)	-	0/29 (0%)	5/28 (18%)	0/28 (0%)	
False father assignment	0/50 (0%)	13/50 (26%)	9/50 (18%)	1/50 (2%)	11/50 (22%)	
True unassigned offspring**	21/21 (100%)	9/21 (43%)	16/21 (76%)	21/22 (95%)	13/22 (59%)	
False unassigned offspring	10/50 (20%)	0/50 (0%)	5/50 (10%)	12/50 (24%)	5/50 (10%)	
Common true father assignment	19/29 (65%)	19/29 (65%)				
Common false father assignment	1/50 (2%)	4/50 (8%)				
Common true UC assignment or true unassigned offspring	9/21 (43%)	8/21 (38%)				
Common false UC assignment or false unassigned offspring	0/50 (0%)	0/50 (0%)				
Common assignment correctness	97%**	91%**				
Total assignment	95% CL		95% CL	95% CL	95% CL	95% CL
True father assignment	0/50 (0%)	41/50 (82%)	23/50 (46%)	0/50 (0%)	22/50 (44%)	
True father as most-likely father	0/29 (0%)	28/29 (97%)	20/29 (69%)	0/28 (0%)	18/28 (64%)	
False father assignment	19/29 (65%)	-	0/29 (0%)	17/28 (61%)	3/28 (11%)	
True unassigned offspring	0/50 (0%)	13/50 (26%)	3/50 (6%)	0/50 (0%)	4/50 (8%)	
False unassigned offspring	21/21 (100%)	9/21 (43%)	19/21 (90%)	22/22 (100%)	18/22 (82%)	
Common true father assignment	29/50 (58%)	0/50 (0%)	7/21 (33%)	28/50 (56%)	10/22 (45%)	
Common false father assignment	-	19/29 (65%)				
Common true UC assignment or true unassigned offspring	-	2/50 (4%)				
Common false UC assignment or false unassigned offspring	-	8/21 (38%)				
Common assignment correctness	-	0/50 (0%)	95%**	-		

*CL: Confidence Level. ** The common assignment correctness includes both types of common assignments to the sampled males and uncollected (UC) male category (PASOS) and the unassigned offspring (CERVUS); at 80% CL, 1/2[19/20+9/9]=0.975 (97.5%), 1/2[19/23+8/8]=0.913 (91.3%); at 95% CL, 1/2[19/21+8/8]=0.952

2.4 DISCUSSION

Several software packages are available for parentage analysis (Newpat, Kinship, PARENTE, PATRI, FAMOZ, PROBMAX, PAPA, PASOS, FamSphere, FAP etc). Paternity inference by parental allocation programs is dependent on several factors: (1) the quality and quantity of markers used; (2) the number of candidate males and other parameters (e.g. relatedness); (3) the number of sampled and unsampled mothers; and (4) the level of confidence required (Slate et al., 2000; present study). CERVUS is very sensitive to the estimate of the proportion of candidate parents sampled (and the total number of candidate males) from the standpoints of the confidence intervals and the accuracy of the paternity assignment. The critical Δ value and the success rate is heavily dependent on the degree of overlap of the pair of distributions of Δ scores which in turn is heavily dependent on the number of candidate males (and the proportion of candidate males sampled). The narrower the overlap, the more paternities can be assigned (Fig 1 and Table 3). The wider the overlap, the fewer paternities can be assigned (Fig 1 and Table 3). The lower the proportion of candidate males sampled, the greater the critical value and the lower the number of paternity assignments (Table 3). Hence, if the number of candidate males (and the proportion of candidate males sampled) cannot be estimated, paternity assignment can be biased. To minimize this bias, we advocate to use a computer programme to estimate the number of candidate parents such as PASOS, Patri, and MasterBayes which uses a Bayesian approach. Alternatively, Araki and Blouin's (2005) provide a mathematical approach to estimate the number of offspring whose parents are not sampled and indirectly the total number of CMs.

This study showed that allocation success declines dramatically as the proportion of candidate parents sampled drops and over-allocation (allocation of offspring bred from unsampled parents to sampled ones) can occur (Table 3). If genotyping were perfect, over-allocation could be avoided by increasing the number of loci, but errors in genetic data are common and increase with the number of loci (Jones and Ardren, 2003). If scoring errors are not tolerated significant losses in the proportions of correct allocations will arise (Gerber et al., 2000). However, tolerating scoring errors and, hence, allowing discrepancies between parental and offspring alleles will lead to a tendency to over-allocate parent-offspring matches. The critical delta increased and the level of confidence decreased when the rate of scoring error increased (Figure 2). As with earlier studies (Hoffman and Amos, 2005), we

advocate calculating the microsatellite genotyping error rate on the studied genetic data set before any analyses by either re-genotyping a portion of the total number of samples or measuring mother-offspring pair mismatches, when mothers are known, prior to any analyses of parentage. If one or more loci show significant higher genotyping error rates than the remaining loci, the analysis should exclude those loci.

Family structure in the population can be problematic for parentage assignment. Activating the relatedness parameter in CERVUS decreased the percentage of false father assignments from 18% to 0% when the known mother was sampled and from 22% to 2% when the known mother was unsampled. However, utilising the relatedness parameter also decreased the percentage of true father assignments from 69% to 66% when the known mother was sampled and from 75% to 57% when the known mother was unsampled. The presence of closely related individuals in the parental files can lead to incorrect assignments and the probabilities of maternal and paternal genotypes become dependent on each other (Hadfield et al., 2006). However, combining assignment of paternity and maternity lowers the bias induced by a related parental population and concerns arise only when the parental population is related to the offspring as full siblings (Thompson, 1976). Thus, a strict exclusion approach and an increase of the number of loci (if necessary) can still enable the assignment of parentage with certainty.

Except for six cases (no mismatches between parents-offspring) in PASOS and seven cases in CERVUS, all false father assignments contained mismatches at up to two loci between the pup and candidate male genotypes and/or the mother-offspring-father triads. True fathers rated most-likely father (with undefined CL) by CERVUS had no father-offspring mismatches (except for one case) but had a Δ LOD score lower than the critical Δ LOD score and hence were not assigned any pups with any defined CL. CERVUS assigned parentage to false fathers (with higher likelihood) with mismatches at one or more loci instead of assigning to the true fathers (with lower likelihood) that had no mismatches (Table 4). A male's likelihood of paternity is positively related to the number of alleles he has in common with the offspring. A homozygous male shares more alleles with a compatible offspring and he will have a higher likelihood of having sired it than a heterozygous male sharing one allele at each locus with the offspring. In addition, parent-offspring pairs that share rare alleles will be assigned with a higher level of statistical confidence than parent-offspring pairs that share

the same number of common alleles, even if they have no mismatches (Neff *et al.*, 2000b). Chapter 3 showed that males with rare genotypes tend to be assigned increased rates of parentage than males with common alleles. Thus, parentage assignment more often goes to the male with the rarer alleles (most often as a heterozygote) and this may bias results towards supporting notion that females prefer more genetically unusual males (Brown, 1997). All common false father assignments contained a high number of offspring-father matching alleles and rare alleles (frequency < 0.1).

2.4.1 Confidence interval

A number of researchers currently report their parental results by saying they have allocated parents with 80% or 95% CL without questioning the meaning and the validity of such statements. The confidence interval indicates the reliability of an allocation or the probability of identifying the correct parent. In CERVUS, the cases with no defined CLs means that the difference between the LOD scores of the two most-likely candidate males or parental pairs is lower than the critical value of Δ , which is heavily dependent on the number of CMs, and suggests that the true father is not in the pool of sampled males. If the number of CMs cannot be estimated accurately, all the confidence intervals and, hence, the assignments should be viewed with great suspicion.

2.4.2 Guideline summary

Incorrect parental assignment can bias estimates of various biological parameters and hence bias ecological and evolutionary interpretations (e.g. female mate choice). The following guidelines derived from the current study may help maximize the probability of correct parental allocation. First, the total number of candidate parents should be reliably estimated using a computer programme (e.g. PASOS or MasterBayes) if the number of the candidate parents is unknown or is inaccurate (Figure 1 and Table 3). Second, the error rate in the studied data files should be determined by re-genotyping a portion of the total number of samples or measuring mother-offspring pair mismatches, when mothers are known, but this measure underestimates the true error rate in the data files (Figure 3). Third, the relatedness level in the study population should be estimated (Figure 1 and Table 3). Forth, the paternity analysis results from two different parental allocation programmes (e.g. CERVUS and

PASOS) should be combined and the common assignment correctness calculated (Table 4). To determine the common assignment correctness of the study system, paternity analyses should be run using simulated genotypes based on the data under study. Our Java programme is available upon request, but has been designed for moderate polygynous systems so may need modification for some studies. Other programmes can generate genotypes based on the allele frequencies of the population under study (e.g. PASOS). The common assignment correctness provides the probability of correct parental allocation and the common true father assignment provides the proportion of true father assignments that has been resolved by the parental allocation programmes (Table 4). Finally, using real genetic data, paternity analysis results from two parental allocation programmes, can be combined with the user retaining the common father assignments, which increases the accuracy of assignments (Table 4). To minimize erroneous assignment decisions, the number of male-offspring pair and/or male-mother-offspring triad mismatches should also be taken into account as well as the number of offspring-parent matching alleles and rare alleles (Chapter 3). We also suggest accounting for demographic, sexual and behavioural information as well as using logic and heuristic methods to develop priors for Bayesian analysis to limit the number of potential offspring-parent relationships considered (Neff et al., 2001, Hadfield et al., 2006).

2.5 ACKNOWLEDGMENTS

This study has been funded by the University of Canterbury (UC) Master's and Doctoral Scholarship and a New Zealand International Doctoral Research Fellowship to SN. We thank Dr. Abigail Caudron (Université de Liège) for the opportunity she gave us to work on the New Zealand fur seal (*Arctocephalus forsteri*). Thank you to Dr. Jon Slate (University of Sheffield) for helping with the CERVUS programme and for reviewing an initial draft of this paper. Special thanks goes to Dr. Pierre Duchesne (Université de Laval) for numerous comments on drafts of this paper. Finally, thank you to Margaret Will (University of Canterbury) and Dr Paul Sunnucks (Monash University) for providing helpful comments on initial versions of this paper.

Chapter Three

Biases in parentage assignment: do heterozygous males achieve greater reproductive success?

Negro S.S.¹, Freeman I.², Gemmell N.J.¹

¹Molecular Ecology Laboratory, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

²Computer Science and Software Engineering, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

Submitted manuscript, please do not quote without first contacting the authors

Abstract

Genetic marker based parentage analysis enables the examination of mating success and social organisation, greatly enhancing our understanding of mating systems. However, such analyses are not without their pitfalls, with erroneous assignments common in open systems (when sampling of parents and offspring is incomplete). This study analysed the effects of the intrinsic bias in likelihood assignment approaches towards assigning higher probability of parentage on individuals with rare alleles and those with heightened offspring-parent matches, which increase with the number of homozygous loci. We showed that, as a consequence of the algorithms employed in the programmes CERVUS and PASOS, heterozygous males with rare genotypes are assigned higher rates of parentage than males with common alleles. Consequently, where two males are both genetically compatible with a given offspring, parentage assignment will more often go to the male with the rarer alleles (most often in heterozygous loci). Thus the commonly used parentage assignment methods may systematically bias the results of parentage analyses towards supporting the notion that females prefer more genetically unusual, most often heterozygous, males. Such a bias may sway investigators towards incorrectly supporting the concept that females choose genetically more unusual males for heterozygosity fitness benefits that underpin the good genes hypothesis, when in fact no such relationship may exist. Incorrect assignment decisions can have a large effect on estimates of various biological parameters and hence bias ecological and evolutionary interpretations. The solutions proposed in this paper can evaluate uncertainty in parentage and population-level parameters, decrease bias in biological parameter estimates, thus increasing the power of parentage assignment.

3 Biases in parentage assignment: do heterozygous males achieve greater reproductive success?

3.1 INTRODUCTION

Molecular ecologists routinely use highly variable nuclear genomic markers, such as microsatellites, to evaluate the fine scale relationships within and between populations. Such analyses are providing major insights into animal mating systems through the elucidation of parentage (Ambs et al., 1999, Gemmell et al., 2001, Foerster et al., 2003, Cohas et al., 2007), social organization (Conrad et al., 1998, Cohas et al., 2006), and sexual selection (Payne, 1979).

Chakraborty and co-workers (1974) published a useful approach for parentage analyses based on exclusion probabilities, but this is not sufficient for parentage inference when multiple males are genetically compatible with each offspring tested; a situation often encountered in populations that are large and have a tendency for site fidelity. In such instances, likelihood-based approaches are preferred and assign paternity to the most likely male if several males are not excluded (Meagher, 1986). Parentage analyses can be performed by a wide range of software packages (CERVUS, FAMOZ, Kinship, Newpat, PAPA, PARENTE, PASOS, PATRI, PROBMAX, FAP, FamSphere, etc), with most of these using a likelihood-based approach to assign paternity to the most likely male.

Three methods are generally used for the assignment of parents to offspring: fractional allocation, categorical allocation, and full probability models (reviewed in Jones and Ardren, 2003). Parentage analysis first utilises demographic, sexual and behavioural data, and heuristics to minimize the number of pedigrees considered followed by the evaluation of the relative likelihood of those pedigrees considered possible. A third step is often included in which population-level parameters are estimated from the pedigree information, such as behavioural groups, mate choice and fecundity (Jones and Ardren, 2003).

Incomplete parental sampling can induce high rates of erroneous assignments, if the number of missing (unsampled) parents cannot be estimated with accuracy (Nielsen et al., 2001,

Duchesne et al., 2005, Chapter 2). Laboratory genotyping errors, mutation, null alleles, and the use of limited number of loci can also result in incorrect assignment (Bernatchez and Duchesne, 2000, Neff et al., 2000b, Hoffman and Amos, 2005). In particular, the presence of closely related individuals in the parental generation is rarely taken into account in parentage analyses. This can lead to incorrect assignments and the probabilities of maternal and paternal genotypes become dependent on each other (Hadfield et al., 2006). However, combining assignment of paternity and maternity lowers the bias that a related parental population induces, so that concerns arise only when the parental population is related to the offspring as full siblings (Thompson, 1976). Chapter 2 investigated the effects of the user-defined parameters (e.g. proportion of candidate parents sampled, genotyping error rate, presence of relatives) on the confidence level and parental assignment in CERVUS v3.0 and PASOS v1.0 (Marshall et al., 1998, Duchesne et al., 2005, Kalinowski et al., 2007). Chapter 2 showed that inaccurate user-defined parameters can lead to erroneous assignments. Two types of assignment errors can arise: failing to assign the correct parent when it is present in the sample and assigning the offspring to a false parent, which can occur when the true parent is absent or present. Few methods have been developed that provide unbiased parentage assignment and an estimation of the relative reproductive success of different groups (Araki and Blouin, 2005, Hadfield et al., 2006). Hadfield and collaborators (2006) develop a class of full probability models that estimate parentage and a wide range of population-level parameters simultaneously by combining behavioural, spatial and genetic data in a Bayesian framework. Araki and Blouin (2005) investigate the effects of the two types of assignment errors on the estimate of relative reproductive success. They show that regardless of assignment method, they can obtain an unbiased estimate of the relative reproductive success and the number of offspring whose parents are not sampled.

Two decades ago, Devlin et al. (1988) showed that the fractional paternity assignment is the most accurate method because it assigns some fraction (between zero and one) of each offspring to all non-excluded candidate parents and seems to circumvent biases intrinsic in the other parentage analysis methods. However, despite this warning categorical parentage assignment is still widely used in molecular ecological investigation, with the parentage allocation programme CERVUS already cited over 1350 times (12th December 2008) on Google Scholar (Marshall et al., 1998, Kalinowski et al., 2007).

Theoretically, every fragment in the offspring's banding pattern is attributable to a fragment found in either of the putative parental patterns, but in the exclusion and categorical approaches, an individual's calculated likelihood of parentage is positively related to the number of matching alleles with the offspring. Thus, a homozygous parent shares more alleles with a compatible, putative offspring, and will have a higher calculated likelihood of producing it than a heterozygous parent sharing one allele at each locus with the offspring. Take for instance a diploid offspring of genotype XY (locus 1), WV (locus 2), GC (locus 3), and a potential parent with genotype XZ (locus 1), WW (locus 2), GC (locus 3). This parent has five matching alleles with the offspring (X, W, W, G and C) and will be assigned a higher likelihood than a putative parent with XZ (locus 1), WU (locus 2), GT (locus 3) that shares only three matching alleles. In addition, parent-offspring pairs sharing rare alleles will be assigned a high level of confidence while parent-offspring pairs sharing common alleles in the population will be assigned with a lower level of confidence, even if they have no mismatches (Neff et al., 2000b, Marshall et al., 1998). Hence, additional weight is deliberately given to rare shared alleles rather than common shared alleles.

Here we investigated the effects of these systematic biases on parental analysis using simulated genotypes. First, we screened homozygous and heterozygous individual genotypes for rare alleles from a wild pinniped population (Chapter 4) with an intermediate level of genetic diversity (New Zealand fur seal, *Arctocephalus forsteri*, overall polymorphic information content, $PIC \pm S.E.M$, 0.746 ± 0.038 , overall observed heterozygosity, $He_0 \pm S.E.M$, 0.744 ± 0.035). Second, we used a Java programme to generate situations where multiple males are genetically compatible with each offspring. Each simulation comprised one heterozygous male with rare alleles, one heterozygous male with only common alleles, and one male that contained homozygous loci. We also used the simulated genotypes from Chapter 2 that has been randomly generated without forcing the simulated genotypes into different classes and using the allele frequencies from the same NZ fur seal population. Third, we analysed the assignments by two different parentage programmes, CERVUS and PASOS (Marshall et al., 1998, Duchesne et al., 2005, Kalinowski et al., 2007), and examined which male is predicted to be the most likely father by the parental allocation programme. Finally, we discussed how assignment errors affect the estimation of biological parameters such as the variance in reproductive success among individuals and propose solutions to reduce the level of incorrect assignment decisions.

3.2 METHODS

3.2.1 *Genetic diversity and rare alleles*

We screened the genotypes of a well-studied New Zealand fur seal, *Arctocephalus forsteri*, (n=157) population for rare alleles. We considered all alleles with a frequency equal or lower than 0.1 to be rare alleles. We calculated the genetic variation at the nine microsatellites used in the NZ fur seal population. We binned all loci into four categories: (1) homozygous locus with common alleles (frequency > 0.1), (2) homozygous locus with rare alleles (frequency ≤ 0.1), (3) heterozygous locus with both alleles common, and (4) heterozygous locus with one or both alleles rare. Then, we calculated the locus-specific and overall proportions of each category to show which categories of loci carry the highest proportion of rare alleles.

3.2.2 *Simulated genotypes*

Parentage inference in large populations often involves situations of multiple males that are genetically compatible with each offspring tested. Slate et al. (2000) retrospectively examine the accuracy of the paternity allocation by CERVUS using a panel of 84 microsatellites in a population of red deer. They notice that most deer were involved in more than one father-(mother)-calf comparison. This study used the genetic data in Chapter 4 which examined the distribution of paternity success in a polygynous NZ fur seal population. One third of the offspring assignments were involved in multiple father-(mother)-pup comparisons (i.e. between two and four compatible males per pup). A detailed analysis of the genotypic quality of the assigned fathers (i.e. father-offspring rare and common alleles, and father-offspring matching alleles) in Chapter 4 showed that father genotypes have up to four rare alleles shared with the offspring and up to four homozygous loci. We used the terminology "extra matching alleles" throughout this Chapter when the number of offspring-parent matching alleles exceeds the number of dinucleotide microsatellites.

We created a Java programme (available on request) to generate situations where several males are genetically compatible with each offspring, using the allele frequencies observed in the NZ fur seal population studied in Chapter 4 (Table 1). For each offspring, three males genetically matched the offspring. One male contained up to four homozygous loci bearing

only common alleles (homo-common, Ho), one male was heterozygous containing only common alleles (hetero-common, Hc), and one male was heterozygous containing up to four shared rare alleles with the potential (hetero-rare, Hr). For each offspring, the programme chose at random whether the "true" father will be homo-common, hetero-common or hetero-rare (considering a diploid individual, allele A represented the first allele and allele B the second allele of an individual locus genotype). The design of the parental reconstruction is described in a flow chart (Figure 1). Finally, the programme generated the "fake", but genetically compatible fathers (with the offspring, but not necessarily with the known mother) using a random assortment of A and B alleles from the offspring.

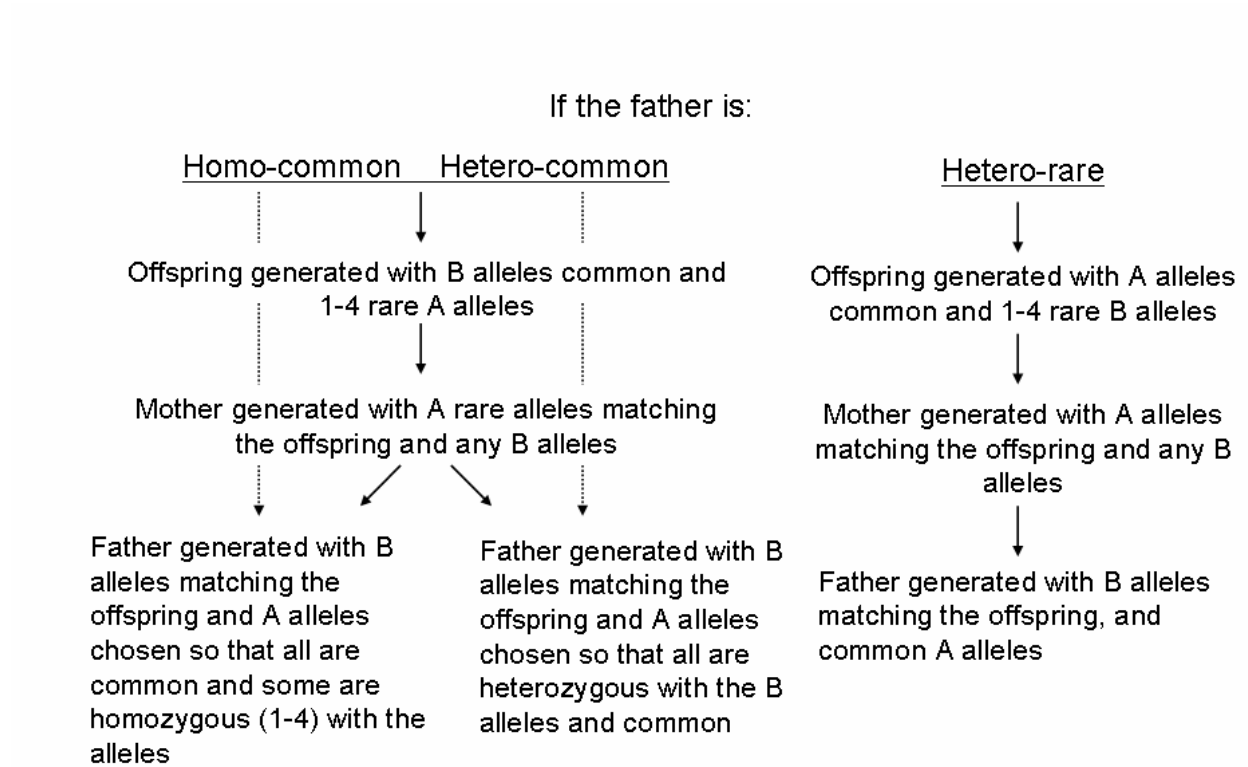
Genotyping errors in genetic data are common and increase with the number of loci scored (Jones and Ardren, 2003). While generating genotypes using our Java programme, we included an error model where the transmission probability from an offspring allele X to a parental allele X is 0.98 and the remaining 0.02 is randomly distributed over all remaining parental alleles where the offspring allele X is misread for another allele. It is well known that the probability of scoring error decreases with the increase of the allelic distance from the focal parental allele (Duchesne and Bernatchez, 2007). In order to incorporate the above error probability distribution observed in real genotype files, we included a restricted error model in the Java programme where the errors were distributed over close neighbours of the parental allele. The allelic distance structure was a function of allelic distance measured in microsatellite repeat units from the focal parental allele X and was designed as follows: X-2 offsets, X-1 offset, X, X+1 offset, X+2 offsets. The transmission probabilities calculated using a 2% error rate were 0.002, 0.08, 0.98, 0.08, 0.002.

The simulated genotypes provided a data set that allowed us to examine which male is predicted to be the father by the parentage programmes, CERVUS and PASOS, and how often the true father is assigned parentage. We also used randomly simulated genotypes that were based on the same genetic data, but not forced into the three male categories (Chapter 2) to test how a natural population behaves in parentage analysis in the face of extra matching alleles and shared rare alleles.

Table 1: Genetic diversity at nine microsatellite loci of the NZ fur seal, *A. forsteri*. A_N : allele number; He_O & He_E : observed & expected heterozygosity; F_{IS} : inbreeding coefficient; PIC : polymorphic information content; HW : Hardy-Weinberg disequilibrium (S: significant, i.e. $p < 0.05$ after sequential Bonferroni correction for multiple tests, NS: non significant). P Non-Excl (1): non-exclusion probability when only one parent is known; P Non-Excl (2): probability of non-excluding two putative parents; P Non-Excl (3): non-exclusion probability of parent pair.

Locus	A_N	He_O	He_E	F_{IS}	PIC	HW	Null allele freq.	P Non- Excl (1)	P Non- Excl (2)	P Non- Excl (3)
NZFS (n=157)										
Hg6.1	10	0.778	0.800	+0.0589	0.827	NS	+0.0285	0.536	0.361	0.184
HI16	13	0.793	0.829	+0.04	0.802	NS	+0.0197	0.535	0.360	0.182
Lc5	6	0.724	0.774	+0.0511	0.734	NS	+0.0321	0.627	0.449	0.269
Hg4.2	18	0.860	0.858	+0.0012	0.845	NS	-0.0076	0.409	0.256	0.090
Pv11	11	0.683	0.657	-0.0487	0.629	NS	-0.0228	0.725	0.537	0.325
M11a	18	0.793	0.817	+0.0216	0.794	NS	+0.0115	0.511	0.340	0.154
Hg6.3	9	0.731	0.791	+0.0868	0.758	NS	+0.0418	0.580	0.402	0.217
Pv9	11	0.827	0.844	+0.0192	0.822	NS	+0.0076	0.509	0.338	0.159
3 E 3	5	0.503	0.539	+0.0641	0.500	NS	+0.0377	0.869	0.716	0.550
Overall	10.8	0.744 \pm 0.035	0.768 \pm 0.035		0.746 \pm 0.038			0.006998	0.000266	0.000001

Figure 1: Design of the parental reconstruction using allele frequencies observed in the New Zealand fur seal population for each three categories of males.



3.2.3 *Parentage programmes*

We chose CERVUS and PASOS, two different programmes that utilise a likelihood framework. CERVUS is widely employed for parentage analyses by the molecular ecological community. Both programmes allow for genotyping errors and incomplete sampling in open systems (where offspring and candidate parent sampling is incomplete). In addition, CERVUS and PASOS have a variety of unique features that aim to minimize the effects of the field, biological and laboratory errors that affect studies of parentage.

CERVUS (Marshall et al., 1998, Kalinowski et al., 2007) assigns parentage to the most-likely individual and parental pair with a known level of statistical confidence (probability of assignment correctness). PASOS (Duchesne et al., 2005) combines an approach based on parental pair likelihoods with a filtering procedure that tolerates errors up to some specified number of offsets, the Maximum Offset of Tolerance (MOT, maximum allelic distance measured in microsatellite repeat units from the focal parental allele). The features of CERVUS and PASOS are summarized in Chapter 2. CERVUS first runs simulations based on user programme settings to generate the critical values of delta (difference between the log-likelihood ratio of the first and second most-likely candidate parents). Then, CERVUS uses the critical value of delta as a criterion for assigning the most-likely parents at defined confidence level. The CERVUS simulation parameters take into account the number of candidate males (here, 300), the proportion of candidate males sampled (here, 1), missing genotypes (here, 0), and genotyping errors (here, 0.02). PASOS has only one user-defined parameter, the MOT, and we set it at 2 as recommended by Duchesne and Bernatchez (2007). The assignment rule in CERVUS reports both offspring-male pair and offspring-mother-male triad on defined confidence intervals (80% or 95%). The assignment rule in PASOS reports the most-likely parental pair (with a defined correctness rate, probability that any assignment is correct) among all potential pairs of sampled and unsampled (uncollected) parents.

3.2.4 *Assignment analysis by CERVUS and PASOS*

We performed a paternity test with known sampled mothers using CERVUS (using both types of simulated genotypes). We also performed a parental pair analysis with unknown

sampled mothers and fathers in CERVUS and PASOS using both types of simulated genotypes. For each offspring, we analysed the assignment from both programmes and reported which category of males (i.e. homo-common, Ho; hetero-common, Hc; and hetero-rare, Hr) was assigned to each offspring. In addition, we analysed the patterns between the number of parentage misassignments and the number of extra matching alleles and shared rare alleles.

3.3 RESULTS

3.3.1 Genetic diversity and rare alleles

We screened the genotypes for rare alleles from a wild population of *A. forsteri* (Chapter 4) with an intermediate level of genetic diversity (Table 1). The microsatellite characteristics are summarized in Table 1 (only one microsatellite, Hg1.4, was in Hardy-Weinberg disequilibrium with an excess of homozygous). We classified the loci into four categories (1) homozygous locus with common alleles, homo-common (frequency > 0.1), (2) homozygous locus with rare alleles, homo-rare (frequency ≤ 0.1), (3) heterozygous locus with both alleles common, hetero-common, and (4) heterozygous locus with one or both alleles rare, hetero-rare. In the studied intermediate genetic diversity population (*A. forsteri*), the hetero-rare loci class predominated (39%) followed by the hetero-common loci class (35%) (Table 2). The homo-rare class is negligible and hence we excluded this category from the parentage analyses using simulated genotypes that have been generated under the conditions described in Figure 1 (Table 2).

Table 2: Microsatellite locus-specific and overall proportions of each locus category (homozygous locus with only common alleles, homozygous locus with rare alleles, heterozygous locus with only common alleles, and heterozygous locus with at least one rare allele) in the studied wild NZ fur seal population. We considered all alleles with a frequency ≤ 0.1 to be rare.

Locus	Homozygous		Heterozygous	
	common	rare	common	rare
<i>A. forsteri</i> (n=130)				
Hg6.1	22%	1%	62%	15%
HI16	22%	1%	63%	14%
Lc5	27%	-	60%	13%
Hg4.2	9%	5%	7%	79%
Pv11	28%	-	20%	52%
M11a	16%	2%	30%	52%
Hg6.3	28%	-	24%	48%
Pv9	18%	3%	24%	55%
3 E 3	50%	2%	22%	26%
Overall	25%	1%	35%	39%

3.3.2 Assignment analysis by CERVUS and PASOS

Both programmes were used to perform parentage testing and analyse the extend of the effects of the parentage assignment biases (i.e. extra matching allele and rare allele biases) using both types of simulated genotypes. We performed paternity and parent pair analyses using CERVUS. We used only PASOS for the parent pair analysis as this programme was originally designed for parentage research in open systems where both parents are unknown. Paternity assignment rates were 46% at 95% CL and 94% at 80% CL, and the parent pair assignment rates in CERVUS were 28% at 95% CL and 60% at 80% CL (Table 3). The correctness rate for any assignment in PASOS was 74% and the parent pair assignment rate was 99% (Table 3). The misassigned paternities and parent pairs are also shown. We found 20 common misassigned parent pairs in CERVUS (80% CL) and PASOS (out of 21 and 52 misassignments in CERVUS and PASOS, respectively) using simulated genotypes that represented the males of each of three categories: homo-common (male containing up to four homozygous loci bearing only common alleles), hetero-common (heterozygous male containing only common alleles), and hetero-rare (heterozygous male containing up to four shared rare alleles with the potential). In contrast when we used simulated genotypes generated randomly without conditions on the father's genotype (Chapter 2) we identified one common misassigned parent pair in CERVUS (80% CL) and PASOS (out of 2 and 31 misassignments in CERVUS and PASOS, respectively). We recorded the number of misassigned males of each male category and analysed the true fathers for each misassigned category (Table 4). We found 10 homo-common and 10 hetero-rare common misassigned males for the parental pair analysis in CERVUS (80% CL) and PASOS. In CERVUS (80% CL), 34% of offspring that were assigned paternities missed out on being assigned parental pairs. Theoretically, critical values of delta are higher for parental pair assignment than for paternity assignment as neither parent is known in the parental pair analysis. Another factor that influences the critical values of delta is whether the sex of the parental genotype is specified prior to parental pair analysis. We measured the "true" and observed (i.e. including true and false assignments by CERVUS and PASOS) heterozygosity and homozygosity levels of parental assignment (Figure 2). The differences between heterozygosity (H_e) and homozygosity (H_o) levels of parental assignment of the true and observed assignment rates differ slightly. However, the differences between hetero-rare (H_r) and hetero-common (H_c) levels of parental assignment of the true and observed assignment rates differ widely. In

CERVUS paternity analysis and PASOS parental pair analysis the H_o levels increased slightly while the H_e levels decreased slightly compared to the true H_o and H_e levels. In CERVUS parental pair analysis both H_o and H_e levels decreased compared to the true H_o and H_e levels. Considering just assignment of fathers, in all parentage analyses the H_r increased and the H_c decreased widely compared to the true H_r and H_c levels of parentage assignment (Figure 2). The breakdown of maternal genotypes that contributed to parental pair assignment is also shown in Figure 2. Finally, the patterns between the number of parentage misassignments and the number of extra offspring-parent matching alleles and shared rare alleles are shown in figure 3 using the simulated genotypes that generated three categories of males (A) and the simulated genotypes generated without forcing them into these categories (B). The parentage assignment increased when the number of extra matching alleles and shared rare alleles increased.

Table 3: Predicted (simulated) and observed parentage assigned by CERVUS and PASOS. The analyses are based on two types of simulated genotypes: (1) genotypes that were categorized (Figure 1) and (2) genotypes that were generated randomly using the same genetic data source. The confidence level and critical delta values are shown for CERVUS and the overall correctness rate (probability that an assignment is correct) is shown for PASOS. The misassigned paternities and parent pairs are shown for CERVUS and PASOS.

CERVUS									
(1) Analysis based on the genotypes simulated into the three categories of males									
Confidence level	Critical delta		Paternities assigned		Misassigned paternities		Predicted parent pairs		Misassigned parent pairs
	Father (known Mother)	Father alone	Parent pair	Paternities assigned	Misassigned paternities	Predicted parent pairs	Parent pairs assigned		
95%	1.37	2.98	2.52	73 (73%)	46 (46%)	8 (8%)	47 (47%)	28 (28%)	6 (6%)
80%	0	1.14	0.66	100 (100%)	94 (94%)	43 (43%)	80 (80%)	60 (60%)	21 (21%)
(2) Analysis based on the genotypes simulated that are not forced into the three categories of males									
95%	1.45	3.27	6.96	61 (61%)	45 (45%)	7 (7%)	19 (19%)	16 (16%)	0 (0%)
80%	0	0.51	4.12	78 (78%)	61 (61%)	20 (20%)	34 (34%)	30 (30%)	2 (2%)
PASOS									
(1) Analysis based on the genotypes simulated into the three categories of males									
Correctness rate for any assignment									
74%	-	-	-	-	-	-	99 (99%)	99 (99%)	52 (52%)
(2) Analysis based on the genotypes simulated that are not forced into the three categories of males									
90%	-	-	-	-	-	-	92 (92%)	91 (91%)	31 (31%)

Table 4: Percentage of misassigned males in each male category (Hr, heterozygous with rare alleles; Ho, mostly homozygous with only common alleles; Hc, heterozygous with only common alleles) and true fathers for each misassigned category.

CERVUS: Paternity			
Misassigned	True father		
80% CL *	Ho	Hc	Hr
16% Hr	5%	11%	-
17% Ho	7%	10%	-
10% Hc	2%	7%	1%
Overall: 43%			
95% CL			
4% Hr	2 (2%)	2 (2%)	-
4% Ho	-	4 (%)	-
-	-	-	-
Overall: 8%			
Cervus: Parental pair analysis			
80% CL			
11% Hr	4%	5%	2%
10% Ho	5%	5%	-
-	-	-	-
Overall: 21%			
95% CL			
4% Hr	2%	2%	-
2% Ho	-	2%	-
-	-	-	-
Overall: 6%			
PASOS: Parental pair analysis			
23% Hr	8%	13%	2%
20% Ho	6%	12%	2%
9% Hc	3%	5%	1%
Overall: 52%			

*CL: confidence level

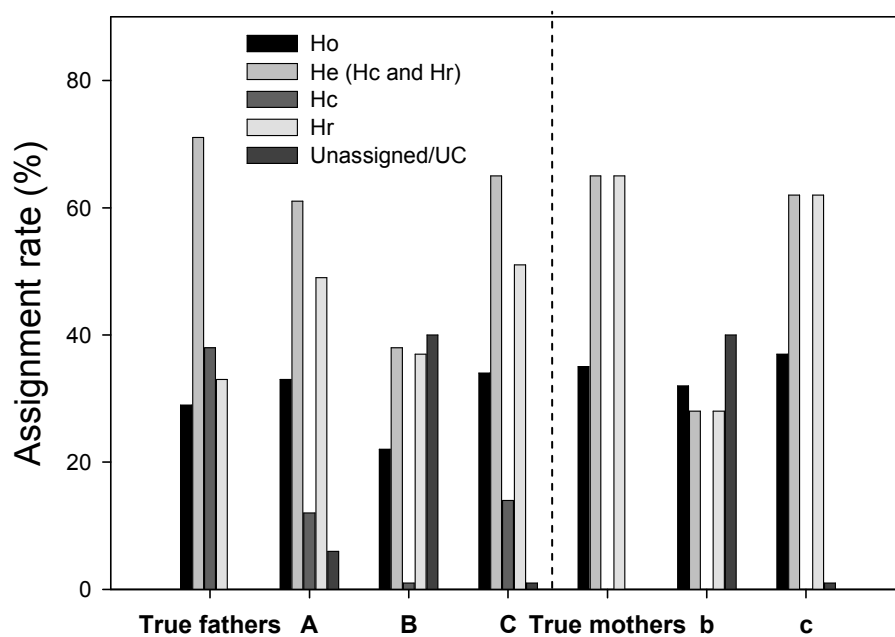


Figure 2: "True" and observed parental inference. CERVUS paternity analysis (A); CERVUS parental pair analysis at 80% CL (B, father assigned; b, mother assigned); PASOS parental pair analysis (C, father assigned: b, mother assigned). The data shown are the true and observed heterozygosity (He) and homozygosity (Ho) levels of parental assignment, the true and observed assignment rates of the heterozygous males containing only common alleles (Hc) and heterozygous males containing 1-4 shared rare alleles (Hr). The rate of unassigned males/females in CERVUS and assignment to the uncollected male/female category (UC) in PASOS is also shown.

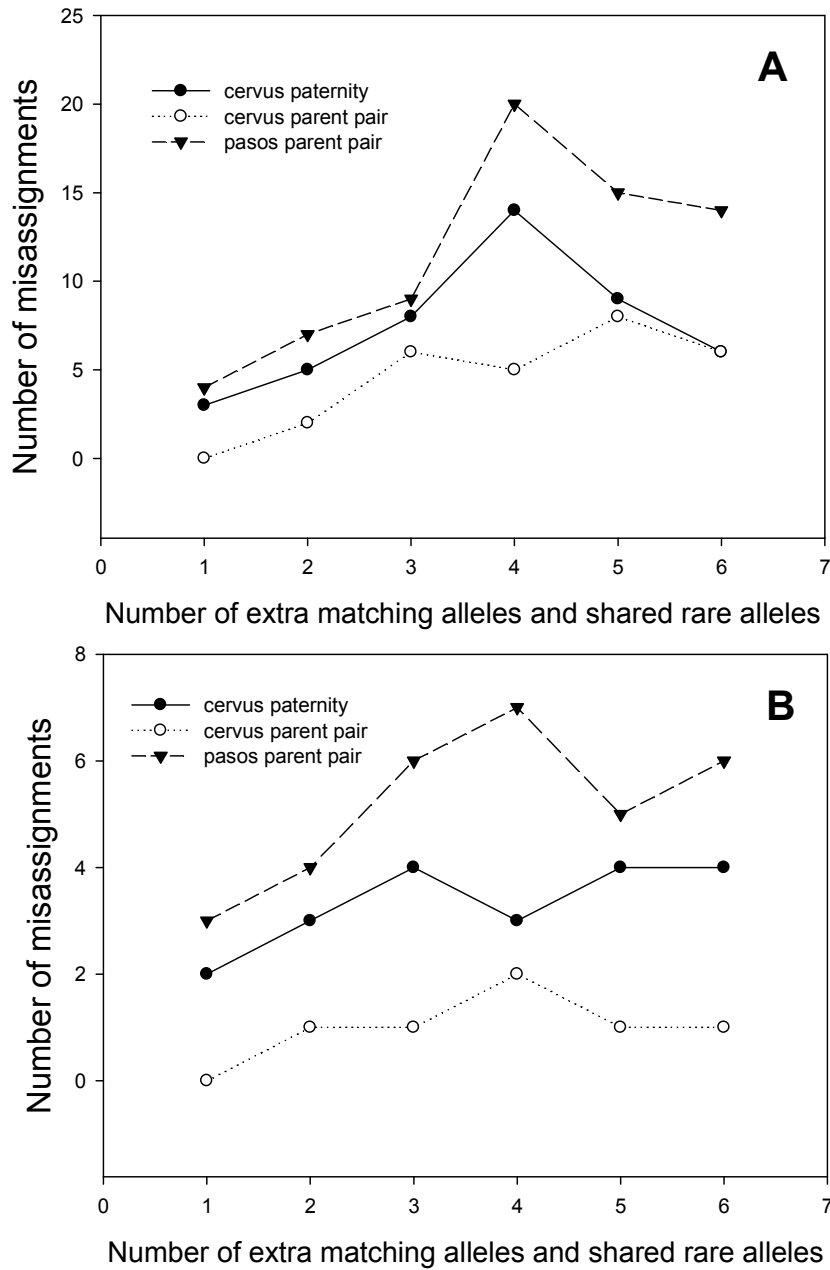


Figure 3: Parentage misassignments as a function of the number of extra offspring-parent matching alleles and shared rare alleles using two parental inference programmes, CERVUS and PASOS, and two kind of simulated genotypes, one forcing the genotypes into three categories of males (homo-common, hetero-common, and hetero-rare) (A), one without forcing the genotypes into any categories (B, Chapter 2).

3.4 DISCUSSION

Parentage analysis enables the examination of mating success and social organisation, greatly enhancing our understanding of mating systems. However, such analyses are not without their pitfalls with erroneous assignments common when sampling of parents and offspring is incomplete. This study analysed the effects of the number of offspring-parent matching alleles and rare alleles on parentage assignments by two fundamentally different parentage programmes, CERVUS and PASOS. We used two types of simulated genotypes: one that forces male genotypes into three categories (homo-common, hetero-common, and hetero-rare), and one in which male genotypes were determined randomly without any conditions on the male's genotype (Figure 1). Genotypes that were constrained into one of the three problematic classes (homo-common, hetero-common, and hetero-rare) were used to test the weighting and influence of extra-matching alleles and shared rare alleles in parentage analysis (Table 4, Figure 2 and 3). The randomly generated genotypes were used to test how parentage assignment methods behave in a natural population when extra matching alleles and shared rare alleles are taken into account.

The observed parentage assignment rates were lower in CERVUS than in PASOS (Table 3). These discrepancies between CERVUS and PASOS are not surprising given that the programmes utilise different algorithms in their likelihood calculations and have different functionalities. The assignment rates that were based on the simulated genotypes constrained into three categories of males were higher than the assignment rates that were based on the randomly determined genotypes (Chapter 2). One factor that can explain this difference is that in the former case we worked with different data types (i.e. closed system vs open system) (Chapter 2). In CERVUS, the parent pair assignment rates are lower than the paternity assignment rates (Table 3). The critical delta value is the criterion that the most-likely males have to fulfil to be assigned with a defined confidence level (80% or 95% CL). The higher the critical value, the lower the assignment rate (Chapter 2). The critical delta values for the parent pair analysis are higher than the critical delta values for the paternity analysis. Hence, higher assignment rates were given by CERVUS for the paternity analysis compared to the parent pair analysis.

For all parentage analyses using the simulated genotypes that included the hetero-common category, the small proportion of hetero-common males misassigned were mostly misassigned against other hetero-common males (Table 4, Figure 2). Some homo-common males were misassigned over the true hetero-common and homo-common fathers, while some hetero-rare fathers were misassigned over the true hetero-common and homo-common fathers. The few cases of unassigned true hetero-rare fathers can be explained by a low number of shared rare alleles (≤ 2) and/or high number of extra matching alleles between the offspring and the misassigned false father. The rare alleles and matching alleles weighting constitutes bias only when the number of shared rare alleles and extra matching alleles are over-weighted to the point it consistently falsely assigns parentage. This study showed that the number of parentage misassigned increases with the number of extra matching alleles and shared rare alleles (Figure 3). The large differences between hetero-rare and hetero-common levels of parental assignment in the true and observed male assignments suggest that many parentage analyses whose aim is to interpret mate choice through the rare-male hypothesis will lead to biased estimates if the assignment decision is not taken under certain criteria (Figure 2, Table 4). The extent and direction of the offspring-parent matching allele and rare allele biases might be dependent on the genetic variation of the studied population. In a low genetic diversity population of NZ sea lion (*P. hookeri*) the hetero-common and homo-common categories predominated (unpublished data). Parentage analysis by computer programmes that utilise categorical methods might lead to stronger bias towards the homo-common category for this population. However, parentage analysis performed from a high genetic diversity population might lead to stronger bias towards the hetero-rare category.

Categorical allocation methods perform weakly when the genotypes from the sample are insufficient to identify parent-offspring relationships with high certainty. Not only do these methods fail to integrate uncertainty in the population-level parameters induced by the uncertainty in the parentage assignments, but they also fail to use information that population-level parameters provide on parentage assignment. Hadfield et al. (2006) show that bias and reduction in power arise from these failures. Incorrect assignment decisions can affect the estimate of quantitative genetic parameters such as gene flow, variance in reproductive success of different groups, or the magnitude of the selection gradients (Morgan and Conner, 2001, Burczyk et al., 2006). Araki and Blouin (2005) applied their assignment methods to a wild population of steelhead trout and show that the assignment error rates can

be high (22.5% and 31.9% when no mismatch is allowed and when up to two mismatches are allowed respectively) in real data. The evaluation of the relative likelihood of the pedigrees considered possible by the parentage programmes is followed by the user-assignment decisions. If no criteria for the assignment decisions are set, the parameter estimates produced can be biased towards those that would be observed under random mating and towards the rare-male hypothesis (Araki and Blouin, 2005, Hadfield et al., 2006, Figure 2 and 3). For example, a common parameter of interest in many parentage analyses is the estimation of the level of extra-pair paternity and genetic benefits of female promiscuity in socially monogamous species (Masters et al., 2003, Cohas et al., 2007, Fossoy et al., 2007). Another common goal of many parentage analyses is to give ecological and evolutionary interpretations for apparent biases in female mate choice in polygynous species (Hoffman et al., 2007). In such studies, naively considering all possible fathers as equally likely will inevitably lead to considerable biases towards extra-pair paternities if the true father is more likely to be the social father in socially monogamous systems or the territorial father in polygynous systems.

Importantly, the systematic biases we describe here towards increased parental assignments for individuals with rare alleles, may sway investigators towards supporting the concept that females choose genetically more unusual males for heterozygosity fitness benefits that may underpin the good genes hypothesis (Brown, 1997, Kempenaers, 2007, Mays et al., 2007). Many studies have reported such associations (Masters et al., 2003, Cohas et al., 2006, Hoffman et al., 2007), often in journals of high impact. It may well be that these studies report true instances where females prefer more genetically unusual males, but where such relationships are marginal it may be a possibility that the relationship has emerged from the intrinsic biases inherent in the parentage programme used.

We propose four solutions to the above problem. If multiple males are genetically compatible with an offspring tested, we first advocate accounting for demographic, sexual and behavioural information as well as logic and heuristic methods treated as prior information in a Bayesian analysis to limit the number of potential offspring-parent relationships considered (Neff et al., 2001). We advocate following the recommendations described in Chapter 2 when running parentage analysis. The number of male-offspring pair and/or male-mother-offspring triad mismatches should be taken into account. In the context of the previous

example in monogamous systems, a common approach is to only consider extra-pair paternities if the social father mismatches at two or more loci out of approximate 9 loci (Fossoy et al., 2007). Finally, this study showed that the number of extra offspring-parent matching alleles and shared rare alleles should be taken into account. In the context of the socially monogamous and polygynous systems described above, a cautionary principle should apply, which is to be suspicious of extra-pair paternities when the extra-pair males have several shared rare alleles and/or extra matching alleles (Figure 3, ≥ 3) with a potential offspring. Note that screening additional loci may increase the confidence with which parentage is assigned, except in cases where candidate parents are highly related (Slate et al., 2000).

Incorrect assignment decisions can have a large effect on estimates of various biological parameters and hence bias ecological and evolutionary interpretations. Considering the four solutions described above can decrease bias in biological parameter estimation, evaluate uncertainty in parentage and population-level parameters, and increase the power of parentage assignment.

3.5 ACKNOWLEDGMENTS

We thank Drs. Brigitta Kurenbach and Maxine Bryant (University of Canterbury, NZ) for reviewing this article. Thanks to Dr Abigail Caudron (Université de Liège) for the opportunity she gave us to work on the New Zealand fur seal (*Arctocephalus forsteri*). Thank you to Dr Laura Boren, Christopher Muller, Rachel Lord, Christina Hoefsmit for their assistance in sampling the NZFS. Thank you to The New Zealand Department of Conservation for providing us the New Zealand sea lion tissue samples. Thanks to Drs Louise Chilvers, Ian Wilkinson, Aurélie Castinel and all the field assistants for their help in the sea lion sampling 2004. SN has been supported by University of Canterbury (UC) Masters and Doctoral Scholarships and a New Zealand International Doctoral Research Scholarship as well as by the Université de Liège (foundation Camille Hela). The work is funded via a contract to NG from the Landcare Research Sustaining and Restoring Biodiversity OBI.

Chapter Four

Alternative mating tactics in the New Zealand fur seal *Arctocephalus forsteri*: When non-territorial males are successful too.

Caudron A.K.^{1,2}, Negro S.S.², Fowler M.³, Boren L.², Robertson B.C.², Poncin P.¹ &
Gemmell N.J.²

¹Behavioural Biology Unit, Zoology Institute, University of Liège, 22 Quai Van Beneden, 4020 Liège, Belgium

²Molecular Ecology, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

³Department of Biology, Sonoma State University, 1801 E. Cotati Avenue, Rohnert Park, CA 94928, USA

Submitted manuscript, please do not quote without first contacting the authors

ABSTRACT:

In polygynous mammals, socially dominant males usually achieve the greatest reproductive success. When the status of many males (genetics, age, size, health, etc.) does not allow them to have a high social rank, theory predicts selection for alternative mating tactics, such as sneaking. Alternative tactics were suggested to explain discrepancies between mating and paternity successes in several species of pinnipeds. However, information on alternative tactics in fur seals is limited and controversial. Here, we focus on the polygynous New Zealand fur seal *Arctocephalus forsteri*, predicting that 1) competition for females is likely to cause a diversification of male mating tactics; and 2) that non-territorial tactics can yield reproductive success. We describe the behaviour of 38 males during 330 h of observation in a large colony. Paternity success is assessed by microsatellite genotyping, using two different programs CERVUS and PASOS, from a pool of 82 pups sampled at the study site and at neighbouring breeding areas. To see if size is correlated with mating tactic, the length of 17 study males was estimated by photogrammetry. Cluster analysis indicates three male behavioural profiles; one corresponds to large territorial males and two illustrate a continuum of alternative tactics employed by smaller non territorial males. Out of the 13 pups that are assigned a father, 62% were sired by 3 territorial males (who accounted for 21% of all the pups born at the study site) and 38% were sired by non-territorial males. Our study highlights that holding a territory is not a necessary condition for reproductive success in otariids.

4 Alternative mating tactics in the New Zealand fur seal *Arctocephalus forsteri*: When non-territorial males are successful too

4.1 INTRODUCTION

In polygynous systems, reproductive success is typically more variable in males than in females and paternal care is limited. Competition between males is strong, with the more competitive males achieving more matings than their rivals (Darwin, 1871, Trivers, 1972, Emlen and Oring, 1977). Depending on their individual status, only a proportion of all males gains reproductive access to females. Status is determined by conditional factors such as genetics, ontogeny, age, size, disease and pathogen load. When differences in status create differences in male relative fitness, selection favours the development of status-dependent alternative mating tactics, within the male mating strategy hence defined as conditional (Gross, 1996). Similarly, Shuster and Wade (2003) predict that in strongly polygynous systems, sexual selection can favour alternative mating tactics in males whose status does not favour competitive abilities. Such alternatives (e.g. sneakers, satellites, helpers) have been observed in many vertebrate taxa, including mammals (Rasa, 1989, Clark et al., 1997, Hogg and Forbes, 1997, Soltis et al., 1997, Linklater et al., 1999, Coltman et al., 1999a, Heckel and von Helversen, 2002).

Among mammals, pinnipeds have been traditionally used as models to study polygyny. They are marine feeders, but come onshore for parturition and postnatal pup care (Bartholomew, 1970). Their often intense territoriality and impressive harems are classic examples of extreme polygyny (e.g. Bartholomew, 1952, Bartholomew, 1953). Molecular techniques to quantify paternities have been introduced with success to investigate the primary mating tactic in a number of species. But despite considerable differences in individual male status induced by sexual selection are observed, very few studies have specifically addressed the presence of alternative mating tactics in pinnipeds. In one such study, Lidgard et al. (2004) compared the success of two out of four male mating tactics they described in the grey seal *Halichoerus grypus* (Sable Island, Canada). The fertilization rate for males using one alternative tactic (i.e. mating with departing females), while lower than for males using the

primary tactic, was significantly higher than zero, indicating the potential fitness value of alternative tactics in a population of polygynous mammals.

Other studies in pinnipeds provide circumstantial evidence for alternative mating tactics. They all highlight a lack of strong correlation between access to females and reproductive success in grey seal *Halichoerus grypus* (Amos et al., 1993, Ambs et al., 1999, Worthington Wilmer et al., 1999), in harbour seal *Phoca vitulina* (Coltman et al., 1998), in northern elephant seal *Mirounga angustirostris* (Hoelzel et al., 1999), and in Antarctic fur seal *Arctocephalus gazella* (Gemmell et al., 2001). In all cases, many pups could not be assigned to the males using the primary tactic, meaning those pups had been sired either by males that did not have apparent access to females (i.e. males using an alternative tactic) or by males that used the primary tactic away from the study site (in the water or at another breeding site). In two more recent studies, improving methodologies (increase sample size: Hoffman et al., 2003, or better matching the pool of males sampled for genetics and for behaviour: Twiss et al., 2006) still left c. 40% of the paternities unassigned to males using the primary tactic. Lancaster et al. (2007) studied the high level of hybridisation involving three species of *Arctocephalus* fur seals on Macquarie Island assigned two hybrid pups to non-territorial males which were hybrids themselves.

Like most pinnipeds, the New Zealand fur seal *Arctocephalus forsteri* is a polygamous, annual colonial breeder. Males come ashore to establish territories at the beginning of the breeding season (austral spring). Females haul out a couple of weeks later to give birth (Mattlin, 1978, Goldsworthy and Shaughnessy, 1994, Boren, 2005). Females are in oestrus about one week after parturition, mate with males and start alternating between foraging at sea and pup nursing onshore. The degree of polygyny ranges from 4 to 10 females per male (Mattlin, 1978).

In this study, we investigated the occurrence of alternative male mating tactics in a large breeding colony of NZ fur seals. Following the arguments of Gross (1996) and Shuster and Wade (2003), we hypothesize that the competition for females leads to a diversification of male mating tactics in this species. We predicted that if non-territorial male fur seals are using alternative mating tactics according to their status, then these males should achieve some reproductive success. We tested these hypotheses by sorting males into objectively

defined behavioural tactics and assessing their success using microsatellite genotyping. We correlated these data with male's body length, as an estimator of male size.

4.2 METHODS

4.2.1 Study site

The Ohau Point breeding colony (42°25'0''S, 173°40'60''E), New Zealand, is a 50-100 m wide and approximately 1 km long colony backed by a steep hill. Its annual pup production in 2003 was about 300 pups (Boren et al., 2006). The substrate of irregular sized and shaped rocks creates small caves and crevices, together with rocky platforms and tide pools. This expanding colony offers fur seals suitable breeding habitat, plenty of space and close access to foraging grounds (Boren et al., 2006). We divided the colony into three areas: Centre (main study site, about 1500 m², i.e. approximately 1/5 of the area of the whole colony and producing about 16% of the colony annual pup production), North and South (the neighbouring breeding areas on each side), each of which are separated by natural landmarks (rocky/boulder ledges) and by non-breeding haul outs (i.e. adults haul-outs with no territories and no pupping).

4.2.2 Behavioural sampling

A total of 330 hours of observation spread over 65 days were conducted in Centre from 30 Oct. 2002 to 2 Jan. 2003 inclusive (covering the whole breeding season). Seals were observed using binoculars and a spotting scope from a cliff-top hide approximately 25 m away from the colony, from 9:00 to 17:00. For most days, 8 consecutive hours of observations were carried out by 2 observers alternating every 2 hours, except when the weather – wind and /or rain – made observations impossible. Days with less than 2 successive hours of observations were not analysed. The potential observer bias was not specifically investigated since both observers were experienced with the study species and site, and regularly calibrated their data collection by overlapping sessions. The total number of males that spent time at Centre during the whole study period was in the range of 50, but it could not be defined exactly since not all males were identified. Out of the males that stayed in the study area long enough to be described using reliable individual features

(approximately 30 minutes), 43 focal males could be identified by natural markings (e.g. flipper scalloping, nose or body scars, McConkey, 1999) or artificial markings (white and yellow road oil-based paint (Resene) applied with a sponge mounted on a 2.5 m metal pole, diameter 1.8 cm). The paint marking was done at the beginning of the season and stopped after the first pup was born in the study area, to reduce disturbance to females. Intrusions in the colony for paint marking males were less than 1 hour per session, by maximum two researchers and successive marking sessions were separated by at least two days without human disturbance, in order to limit the potential impact of marking on males' behaviour. Unlike other taxa (e.g. many bird species), pinnipeds do not use complex body color patterns that could be disturbed by mark paints.

All interactions involving focal males at Centre were recorded (Continuous All Occurrence Sampling, Altmann, 1974) in a time table and categorised as follows: **intra-sexual interactions** involved walking towards, following, chasing, attacking, lunging at, fighting, investigating, or having a low intensity aggressive interaction with another male (e.g. investigating/sniffing followed by an open-mouth threat). For each interaction, the focal male(s) was (were) sorted into aggressive (the male initiating the interaction, the challenger) or submissive (the target, the defending male). **Inter-sexual interactions** involved walking towards, investigating with-holding (herding), attempting to and copulating with a female, accepting female advances (female biting male's neck, mounting on his back, soliciting) and vocalizing to a female (whimpering). **Male displays** involved vocalizations, either to another male or no apparent target, and full-necking (upright display) (see Stirling, 1970, Miller, 1971 for descriptions of behaviours and vocalizations). The location and the time of all interactions were recorded together with the type (or identity, when it was known) of individual(s) focal male(s) interacted with.

Territory sizes were estimated using digital photographs of the study site processed with the software TurboCAD v4 Learning ed. (Fowler, 2003): the limits of individual males' territories, based on their patrolling behaviour and on the location of all their daily intra-sexual interactions, were hand-drawn onto a map of the field site twice a day. Territories were later outlined on PVC sheet and the sheet overlaid on the computer screen displaying the digital photo of the study area corrected to approximate a planar projection. Paint marks on rocks at known distances (visible on the photographs) were used for scaling.

The behavioural profile of study males was quantified using seven variables: 1) the date of arrival (first day seen on the study site) counted from October 30; 2) the total number of days spent on the site (equivalent to tenure duration for territorial males); 3) the estimated area of the territory defended, in square meters (a value of 1 was given to males which only defended their resting spot, but no area around it, nor any female); 4) the mean frequency per hour of aggressive intra-sexual interactions; 5) the mean frequency per hour of submissive intra-sexual interactions; 6) the mean frequency per hour of inter-sexual interactions and 7) the mean frequency per hour of male dominant displays.

4.2.3 *Male size*

The size of a sub-sample ($n=17$) of the focal males was estimated using basic photogrammetry (Baker, 1960, Haley et al., 1991). Body length from the tip of nose to the tip of the tail was measured from digital photographs of individuals lying straight, perpendicular to the objective, using measures of natural rock marks as a scale. Body length (cm) estimated from several photographs of the same individual taken on different days over the season showed a maximum CV of 6.2% ($n=4$ males, with 10 photos taken on different days for each of the 4 males).

4.2.4 *Genetic sampling*

Male skin samples ($n=20$) were collected from a distance using a cross-bow launched skin sampling device (Gemmell and Majluf, 1997). We were unable to sample all focal males, due to male turnover early in the season (November), and then later, to avoid disturbing pregnant females seeking a place to pup (December). To investigate the result of male reproductive efforts the previous year, mother-pup pairs were sampled the following season at Centre (as many pairs as possible), and at the two neighbouring breeding areas (10 to 11 pairs randomly selected at North and at South). For pups, a small piece of skin was taken from the trailing edge of fore flipper using piglet ear notch pliers (Majluf and Goebel, 1992). For mothers, more mobile and skittish than males and pups, a cross-bow launched hair sampling device was custom-designed with sticking dart to pull hair (Caudron et al., 2007). Limitations in the number of females sampled was due to the fact that most were not individually marked and

could only be located when interacting with their identified pup. A female was assumed to be the mother of a pup only if their interaction was long enough to ensure the female did not reject the pup, show any sign of hesitating between several pups or interact in a maternal fashion with another pup. All samples collected were stored immediately in 70% ethanol and then in 90% ethanol one month later.

4.2.5 Genotyping

In order to match mother-pup pairs and putative fathers, we genotyped all samples using ten microsatellite markers. Whole genomic DNA was extracted from skin biopsies using an adapted Chelex 100TM protocol (Walsh et al., 1991) and from hair follicles as described in Caudron et al. (2007). Ten informative loci were amplified (Table 1), run and scored for skin samples (n=111) as described by Robertson and Gemmell (2005). For hair samples (n=45), microsatellite amplification was achieved using PCR conditions described in Caudron et al. (2007) and PCR products were size-fractionated on 6% denaturing polyacrylamide gels using a low concentration alkali salt conductive medium (rapid and high resolution of small DNA fragments, Negro et al., 2006). Samples for which three or more loci did not amplify (n=2 females out of 45 hair samples) were excluded from the dataset.

4.2.6 Identity Checking

All files were checked for duplicate genotypes using the Identity function of CERVUS v3.0 (Marshall et al., 1998, Kalinowski et al., 2007). The probability of an identical multilocus genotype occurring by chance in two unrelated individuals at all ten polymorphic loci was calculated for each locus and across all loci using GenAlEx v6 (Peakall and Smouse, 2006) (Table 2), using the method of Paetkau and Strobeck (1994). In addition, because NZ fur seals tend to be philopatric, as a conservative measure, the probability of identity assuming that all individuals are siblings was also calculated according to Evett and Weir (1998) as implemented in GenAlEx (Figure 1). Duplicate samples (n=3 pups, n=1 female) were excluded from the genotype files prior to analysis.

Table 1. Primer sequences, polymorphism characteristics and literature sources for each of the ten pinniped microsatellite loci used (N: number of alleles).

Locus	Primers sequence (5' → 3')	Allele size	N	Species	Reference
Hg1.4 (1997)	F: CTCCAAAGACGACTGAAACCC R: TACCATAATCTTTGTGGCTCTG	187-209	7	Grey Seal	Gemmell et al.
Hg4.2	F: AATCGAAATGCTGAGCCTCC R: TGATTTGACTTCCCCTTCCCTG	126-188	19	<i>Halichoerus grypus</i> Grey Seal	Allen et al. (1995)
Hg 6.1	F: TGCACCAAGAGCCTAAGCAGACTG R: CCACCAAGCCAGTTCACCCAG	143-166	10	<i>Halichoerus grypus</i> Grey Seal	Allen et al. (1995)
Hg6.3	F: CAGGGGACCTGAGTGCTTATG R: GACCCAGCATCAGAACTCAAG	228-249	9	<i>Halichoerus grypus</i> Grey Seal	Allen et al. (1995)
HI16	F: CACTTATCTCGCCCTATATCCA R: CAGCCACAGCCCAACACAA	135-169	13	<i>Halichoerus grypus</i> Leopard seal	Davis et al. (2002)
Pv9	F: TAGTGTTTGGAAATGAGTTGGCA R: ACTGATCCTTGTGAATCCCAGC	166-186	11	<i>Hydrurga leptonyx</i> Harbour seal	Allen et al. (1995)
Pv11	F: GTGCTGTGAATTAGCCCATTTATAAG R: CAGAGTAAGCACCCCAAGGAGCAG	151-173	11	<i>Phoca vitulina</i> Harbour seal	Goodman (1997)
M11 A	F: TGTTTCCCAGTTTACCA R: TACATTCACAAAGGCTCAA	135-182	18	<i>Phoca vitulina</i> Southern elephant seal	Hoelzel et al. (2001)
3E3	F: GCATACTCTTCACCTACCAATGG R: CTCCAGCTAGCTTCCTCTCTTG	213-223	5	<i>Mirounga leonina</i> Harbour seal	Kappe (1998)
Lc5	F: ATCTTCAGGCTTCTTCT R: TTCACGGACTCAAATAAT	156-169	6	<i>Phoca vitulina</i> Crabeater seal	Davis et al. (2002)
Overall		126-249	10.5	<i>Lobodon carcinophagus</i>	

Table 2. Genetic diversity measures for 10 microsatellite loci used to analyse individuals from the NZ fur seal *A. forsteri* colony at Ohau Point. A_N : allele number; He_O & He_E : observed & expected heterozygosity; F_{IS} : inbreeding coefficient; PIC: polymorphic information content; HW: Hardy-Weinberg disequilibrium (S: significant, i.e. $p < 0.05$ after sequential Bonferroni correction for multiple tests, NS : non significant); PI: probability of identity between two unrelated individuals; P Excl (1): probability of parentage exclusion when only one parent is known; P Excl (2): probability of excluding two putative parents.

Locus	A_N	He_O	He_E	F_{IS}	PIC	HW	Null allele freq.	PI	P Excl (1)	P Excl (2)	Error Rate
Hg6.1	10	0.778	0.800	0.0589	0.827	NS	+0.0285	0.055	0.473	0.822	0
HI16	13	0.793	0.829	0.04	0.802	NS	+0.0197	0.054	0.480	0.829	0
Hg1.4	7	0.521	0.808	0.3636*	0.780	S	+0.2084	0.064	0.444	0.805	-
Lc5	6	0.724	0.774	0.0511	0.734	NS	+0.0321	0.090	0.373	0.731	0
Hg4.2	18	0.860	0.858	0.0012	0.845	NS	-0.0076	0.030	0.579	0.906	0
Pv11	11	0.683	0.657	-0.0487	0.629	NS	-0.0228	0.143	0.272	0.673	0
M11a	18	0.793	0.817	0.0216	0.794	NS	+0.0115	0.056	0.475	0.834	0.0501
Hg6.3	9	0.731	0.791	0.0868	0.758	NS	+0.0418	0.075	0.413	0.776	0
Pv9	11	0.827	0.844	0.0192	0.822	NS	+0.0076	0.044	0.521	0.861	0
3 E 3	5	0.503	0.539	0.0641	0.500	NS	+0.0377	0.252	0.156	0.497	0
Overall	10.8	0.721	0.774		0.747			3.418×10^{-12}	0.996	0.9999	0.0056

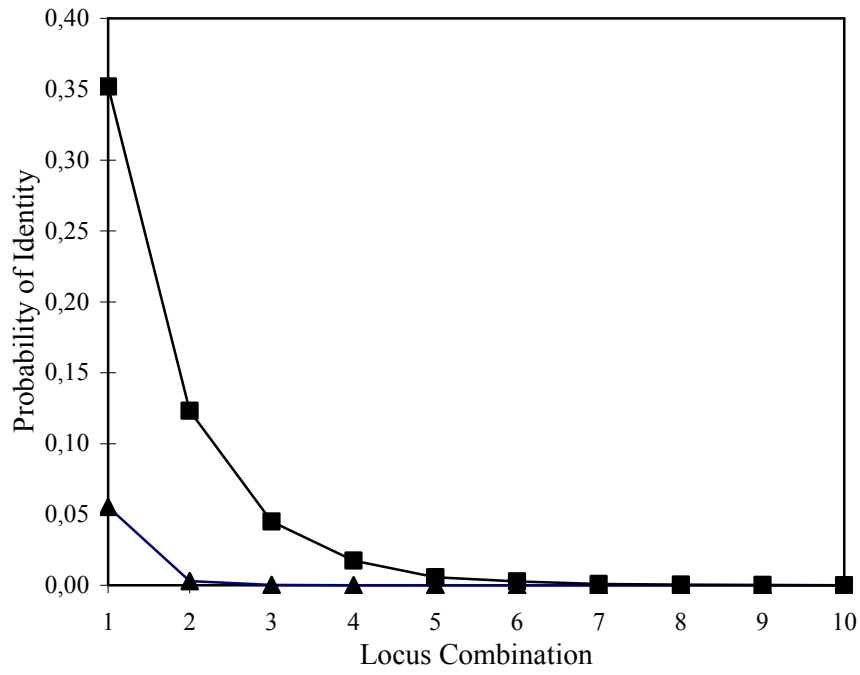


Figure 1. Probabilities of identical genotypes between two unrelated (triangles) and sibling (squares) individuals. Locus combination means cumulative effect of loci.

4.2.7 *User-defined parameters and Paternity Allocation*

Prior to paternity analysis, all ten microsatellites were tested for their appropriateness as population genetic markers. Specifically, we tested for deviations from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) using Genepop v3.4 (Raymond and Rousset, 1995), while null allele frequencies were estimated using CERVUS v3.0 (Table 2). Probabilities of parentage exclusion (i.e. the average capability of a marker system to exclude any given relationship) were calculated according to Jamieson and Taylor (1997) using GenAlEx. Knowing one of the parents increases the likelihood that the assignment of the other parent is correct; therefore the probability of parental exclusion is always lower when only one parent is unknown than when both parents are unknown (Table 2).

We followed the guidelines presented in Chapter 2 and 3 to determine reliable user-defined parameter values to perform paternity testing. Specifically we 1) determined the error rate in the genetic data, 2) determined the relatedness level in the studied population, 3) determined the total number of the candidate males, 4) combined the paternity results from two different parental allocation programmes and calculating the common assignment correctness.

The major cause of mismatches between offspring and their biological parents is through microsatellite genotyping errors (Hoffman and Amos, 2005). To estimate the error rate of our dataset, we checked 43 mother-pup pairs for mismatches using CERVUS. The mean observed error across loci was 0.0142. One pair showed three mismatches at multiple loci, suggesting that the pair being sampled was not genuine. Excluding this pair, the mean observed error across loci was 0.0056. Relaxed parameter analyses (i.e. error rate > 0 in CERVUS and PASOS) will increase assignments to a false father while stringent parameter analyses (i.e. error rate = 0), where laboratory typing errors are not considered, may increase false exclusions of the true father. The relatedness estimator, R (Queller and Goodnight, 1989) for our genetic data, was determined using the programme GenAlEx v6 (Peakall and Smouse, 2006). Among the candidate males sampled, 10 were related with a mean R of 0.450. Finally the total number of candidate males was determined using PASOS v1.0 (Duchesne et al., 2005). The programme estimated the missing portion of males (0.536) and hence calculates the total number of candidate males in the population ($n = 43$).

We performed paternity analyses with two different programmes, PASOS v1.0 and CERVUS v3.0 (Marshall et al., 1998, Kalinowski et al., 2007). PASOS combines an approach based on parental pair likelihoods with a subsequent filtering procedure that tolerates errors up to a number of offsets (Maximum Offset Tolerance MOT) determined by the user. The allocation starts with the search for the most likely pair among all potential pairs of collected parents. We set the MOT at two as recommended by Duchesne and Bernatchez (2007) (Chapter 2). For a valid comparison with CERVUS, we informed PASOS that the mother of a particular offspring is known *a priori* by running a paternity analysis for this particular offspring with a parental file containing a single genotype for the known mother.

CERVUS, which uses a maximum likelihood approach to assign paternity, takes into account scoring error and determines statistical confidence for assigned paternities through simulations that incorporate characteristics of the sample (e.g. size of candidate parents, proportion sampled, rate of typing error, relatives). The confidence levels (CL) are determined based upon the difference in LOD (log of the likelihood ratio) scores between the most likely candidate male and the second most likely candidate male. The paternity analysis results from CERVUS and PASOS were combined and the common father assignments were retained. To minimize erroneous assignment decisions, an analysis on a case-by-case basis has been achieved by taking into consideration mismatched loci between candidate male–offspring–known mother triads and candidate male–offspring dyads (when mother was unknown), the effect of offspring-father matching alleles and rare alleles (Chapter 3). The number of assignments at 95% CL in CERVUS was small due to the high number of candidate males sampled being related among themselves and the mother sample not being available for many genotyped pups, hence confidence level in CERVUS was set at 80% and the results combined to PASOS.

The common assignment correctness (97%) and the proportion of common true assignments that can be resolved by both parental allocation programmes (65%) were calculated in Chapter 2 using simulated genotypes based on the focal NZ fur seal population.

4.2.8 *Statistical analysis*

Agglomerative hierarchical clustering (Johnson, 1967, Aldenderfer and Blashfield, 1984)

was used to sort N individual male behavioural profiles into meaningful, objective classes (Coltman et al., 1999b). Variables were standardised to minimize bias in weighting that may result from differing units and ranges. An N X N matrix was built using the Manhattan (City-block) distance, which examines the sum of the differences between the attributes of pairs of individuals. The matrix was visualised by a tree using Ward's linkage that clusters by assessing the group's variance (StatSoft, 2005, Wishart, 2006). (M)ANOVAs followed by post-hoc Scheffé tests show the significantly different pair-wise comparisons between clusters. Variables were log-transformed as necessary to meet assumptions of parametric statistics (Sokal and Rohlf, 1995). For data that could not be transformed (presented in the results as medians and ranges, instead of means \pm se), non-parametric Kruskal-Wallis tests were used. A χ^2 test was used to compare the expected and the observed reproductive success of territorial and non territorial males. Analyses were done using STATISTICA v7.1 (StatSoft, 2005).

4.3 RESULTS

4.3.1 *Male Behaviour:*

Out of approximately 50 males that passed time at Centre during the study period, 43 were identified. Five of them spent less than three hours each at Centre which wasn't considered enough to describe their behaviour. The breeding behaviour of the other 38 males was described using seven variables (Table 3). A large proportion of study males at Centre (82%) were not territorial. Most of these males were mobile, being observed in the study area at various locations during the breeding season, while the other males tended to be more site-faithful. In addition to size and secondary sexual characteristics, behavioural data suggest that these non-territorial males are non-breeding immature males, territorial males temporarily absent from their territory located outside our study site or males using an alternative tactic.

To distinguish breeding tactics, males were sorted into objective classes, based on similarities in their behavioural profile (StatSoft 2005). Clustering identified three classes of male (Figure 2). Note that the variable "territory area" was not used in the analysis, because it could not be normalised by transformation due to many males being non-territorial and hence

scoring a value of zero for this variable. A MANOVA confirms the three classes significantly differ (Wilks $F=7.3$, $df=12$, $p<0.01$, Table 4). Post-hoc (Scheffé) tests show the following significant differences among all pair-wise comparisons: class 1 males were characterised by a higher frequency of aggressive male-male interactions, of male-female interactions and of dominant displays than for the other classes, and a lower frequency of submissive male-male interactions. All the males of class 1 defended a territory and hence corresponded to the territorial profile. Class 2 males arrived later than class 3 males, and stayed on-site for a fewer number of days than class 3 and class 1 males. Class 2 males were non-territorial visitors (i.e. transient males). Finally, class 3 males arrived earlier and stayed for more days than class 2 males and were non-territorial residents. Males of classes 2 and 3 can be considered as using alternative tactics. Note that males (temporarily) defending only their own body location are found in both classes 2 and 3.

The variable “estimated size” was not included in the cluster analysis as we could only obtain it for a subsample of study males (body length range=122-168 cm, median=151 cm, $n=17$). Size significantly varied between the male classes (Kruskal-Wallis $H=8.05$, $p<0.05$), with class 2 males (transient) having significantly shorter body length than class 1 males (territorial).

Only seven copulations were observed in the study area, over the total study period (330 h). A similarly low number of copulations was observed in all portions of the Ohau Point colony (Boren 2005). In the very broken ground of *A. forsteri* habitat, recording copulations (a usually quiet and immobile interaction) is probably not an adequate index of male mating success. It suggests copulations preferentially take place in concealed areas (crevices, caves), at night or at sea. Maternal attendance data at Ohau Point colony ($n=23$ VHF tracked females, Boren 2005) showed that most females perform overnight foraging trips, providing males with increased opportunities to copulate in near-shore water and/or at night, with departing and arriving females.

Table 3. The breeding behaviour of New Zealand fur seal males at Ohau Point described by seven variables. The variable arrival date is given as mean \pm SE (range) as its distribution does not significantly differ from normality.

Behavioural variables:	median, percentile 25%, percentile 75% (range) unit (n=38 study males)
1. Arrival date (day 1 = 30 oct)	19.8 \pm 2.4 (1-54)
2. Numbers of days on-site	7.5, 25%=4, 75%=16 (2-46) d
3. Mean territory area	0, 25%=0, 75%=1 (0-357) m ²
4. Intrasex. interact. aggressive	0.62, 25%=0.33, 75%=0.98, (0.09-3.23) /h
5. Intrasex. interact. submissive	0.32, 25%=0.10, 75%=0.55 (0-1.63) /h
6. Intersexual interactions	0.71, 25%=0.39, 75%=1.06 (0-3.65) /h
7. Dominant displays	0.21, 25%=0.05, 75%=0.43 (0-2.09) /h

Table 4. New Zealand fur seal study males sorted by hierarchical clustering. A MANOVA on log-transformed data confirms the three classes significantly differ ($F=7.3$, $p<0.01$), results of ANOVA's for each variable are detailed; Kruskal-Wallis was used for body length (only measured in a subsample of study males, could not be normalised). The symbols * and ° indicate the significant differences amongst all pair-wise comparisons (post-hoc Scheffé tests). The variable Arrival date is given as mean \pm SE as its distribution does not significantly differ from normality.

Classification of males (clustering was based on variables indicated by #)	Class 1 Territorial n = 7 males median (range) unit	Class 2 Non-territorial Visitor n = 16 males median (range) unit	Class 3 Non-territorial Resident n = 15 males median (range) unit	Notes
Time observed	107.6 (30.7-285.2) h	7.6 (3.0-35.5) h	46 (14.9-185.2) h	
# Arrival date (day 1 = 30 oct)	14.4 \pm 5.4	28 \pm 3.4 *	12.2 \pm 2.9 *	$p < 0.01$, univariate $F = 5.9$
# Numbers of days on-site	16 (4-46) d *	3 (2-8) d *°	13 (4-34) d °	$p < 0.01$, univariate $F = 12.8$
Mean territory area	79.2 (45.7-357) m ²	na	na	
# Intrasex. interact. aggressive	1.6 (0.7-3.2) /h *°	0.6 (0.1-1.9) /h *	0.4 (0.2-1.4) /h °	$p < 0.01$, univariate $F = 9.4$
# Intrasex. interact. submissive	0.02 (0-0.2) /h *°	0.4 (0-1.6) /h *	0.4 (0.05-1.1) /h °	$p < 0.01$, univariate $F = 15.7$
# Intersexual interactions	1.9 (1.2-3.7) /h *°	0.6 (0-1.5) /h *	0.5 (0.3-0.9) /h °	$p < 0.01$, univariate $F = 5.1$
# Dominant displays	1.1 (0.4-2.1) /h *°	0.1 (0-0.7) /h *	0.2 (0-0.8) /h °	$p < 0.01$, univariate $F = 5.7$
Estimated body length	156.5 (128-168) cm * (n=6)	125 (122-127) cm * (n=3)	149.5 (128-166) cm (n=8)	$p < 0.05$, Kruskal-W H=8.05
Paternity	4 (4) pups *° (n=3)	0.5 (0-1) pups * (n=4)	1 (0-2) pups ° (n=7)	$p < 0.01$, univariate $F = 26.9$

4.3.2 *Characterization of microsatellite loci*

Deviations from Hardy-Weinberg equilibrium (HWE) were assessed using a χ^2 goodness-of-fit test, comparing observed genotype frequencies with expected genotype frequencies calculated from allele frequencies assuming HWE. One locus (Hg1.4) showed a significant deviation from HWE (homozygous excess), even after Bonferroni corrections for multiple comparisons (Sokal and Rohlf, 1995) which could indicate the presence of a null allele at this locus; this locus was then omitted from the parentage testing. The probability of two unrelated individuals having identical genotypes for the nine polymorphic loci was negligible (3.418×10^{-12}) (Table 2). The probabilities of identity between unrelated and sibling individuals for increasing locus combinations suggest identical genotypes were most likely the result of resampling. Duplicate samples were removed from the dataset.

4.3.3 *Reproductive success of study males*

Fifteen males were skin-sampled at Centre (about 30% of all males that stayed at Centre for more than 30 min over the whole study period). One of the males sampled was not included in the behavioural observations as he stayed for less than 3 h. Five additional males were sampled at South (non-Centre males), where no behavioural data were collected. Out of the 15 males sampled at Centre, only 3 were territorial. The 12 others displayed a range of behaviours that included defending only a resting location or no defence at all. The 3 sampled territorial males represented 42.8% of the 7 territorial males in the study area.

Eighty-five pup samples were genotyped, including 41 at Centre (88% of the total pup production at the study site), 25 at South and 19 pups at North. For 42 of these samples, the attending mother was also sampled (mother-pup pairs). Out of those 85 pups, analysis showed 3 were duplicates, so a total of 82 individual pups were genotyped. We could assign a father to 24 (29%) of the 82 genotyped pups based on the common father assignments by CERVUS and PASOS and on the assignment decisions.

Of 38 pups sampled at Centre (41 – 3 duplicates), 12 (32%) were assigned a known father that was observed at Centre in the year of conception and 1 pup was sired by a non-Centre male sampled at South of a total of 5 non-Centre males sampled. Eleven additional pups that were not born at Centre but at neighbouring breeding areas (4 at North and 7 at South) were

assigned a father sampled at Centre.

Out of a total of 20 sampled males, 5 males that were not territorial at Centre sired pups born at Centre in the following season. Attendance data in Centre showed that at least 3 of these males were not long-term territory holders elsewhere neither, because they stayed at Centre for 34, 31 and 20 days respectively, out of 65 observation days. Each of the territorial males sampled at Centre (n=3) sired pups at Centre (8 pups in total, which means 21 % of all pups sampled at Centre were sired by these 3 territorial males). In addition, two Centre territorial males sired 4 non-Centre pups, born in other areas (2 pups each).

For males that were both measured (body length) and genotyped, five out of six males ≥ 152 cm had a pup (i.e. 83% of these “larger” males), while two out of three males ≤ 147 cm had a pup (i.e. 67% of these “smaller” males). Overlaying paternity results and behavioural profiles shows that males of all three behavioural classes were assigned pups (Figure 2): 100% of 3 genotyped class 1 males, 71% of 7 genotyped class 3 males and 50% of 4 genotyped class 2 males sired pups. The observed average reproductive success for the 3 territorial (class 1) males genotyped (2.7 pups/male) was significantly higher than expected, while for non territorial males, it was lower than expected ($\chi^2=14.019$, $df=1$, $p<0.001$; with fifteen males genotyped at the study site and paternities assigned to 13 pups born at the study site, the expected average success was 0.87 pup/male). Nevertheless, out of the 36 identified males that were not territorial at Centre, at least 22% (8 males) had one or two pup(s) (only 12 of the 31 non-territorial males included in behavioural analysis were genotyped).

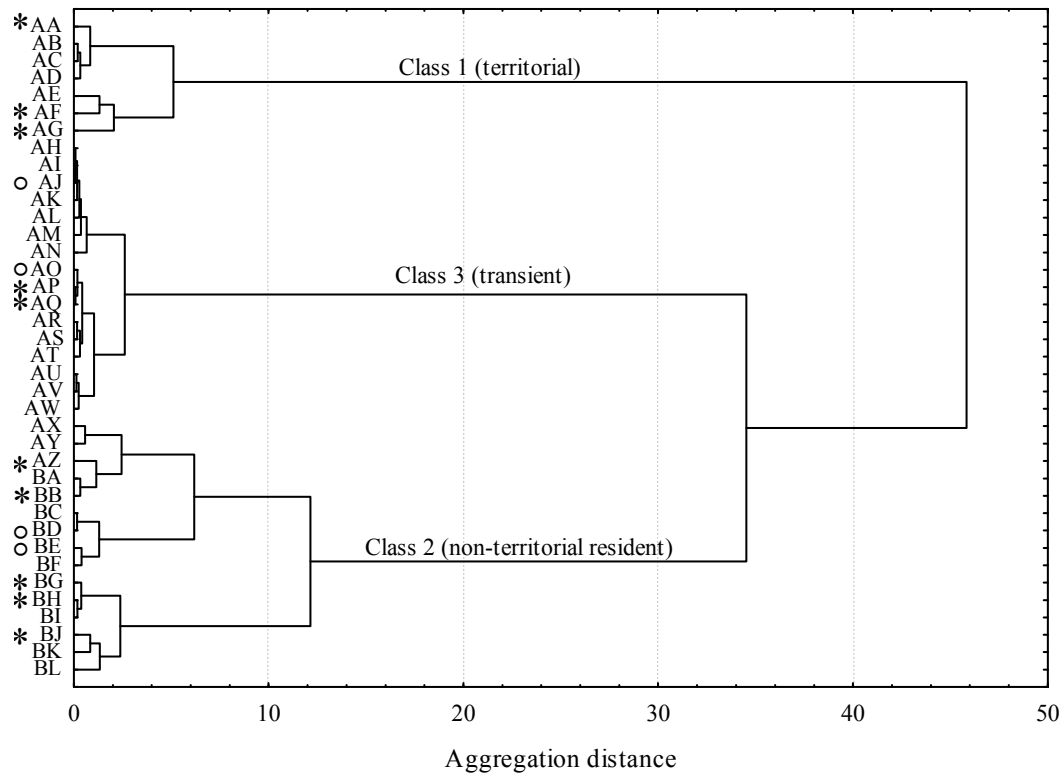


Figure 2. Hierarchical clustering of male behavioural profiles (Manhattan distance, Ward's linkage, StatSoft 2005) suggesting three classes. Class 1 corresponds to territorial males. The other classes can be described as transient and non-territorial resident profiles. Genotyped males that were assigned pup(s) are indicated by *, genotyped males that were not assigned any pup are indicated by °. Class 1 males had a significantly higher frequency of aggressive male-male interactions, of male-female interactions and of dominant displays than other males, and a lower frequency of submissive male-male interactions. Class 3 males arrived later than class 2, and stayed on-site for less days than both class 1 and class 2. Class 2 males arrived earlier and stayed for more days than class 3 males.

4.4 DISCUSSION

By combining ethology and molecular genetics to assess the paternity success of focal males, we found evidence of alternative mating tactics in a large breeding colony of New Zealand fur seals. Despite being limited to a small number of study males and to a single reproductive season, our results clearly show alternatives to territoriality yielding fur seal males some reproductive success.

Twenty-one percent of 38 pups genotyped at our study site were sired by three large territorial males. The observed success of these territorial males was significantly higher than the average expected, in agreement with a skewed male success in polygynous systems: male adopting a primary tactic for a long tenure typically achieve more successful copulations than others (e.g. Boness and James, 1979, Anderson and Fedak, 1985, Arnould and Duck, 1997, Wainstein, 2000). However, our results show that alternative mating tactics can co-exist with the primary territorial tactic and allow many non-territorial males some success. At the scale of this study, holding a territory was not a necessary condition for male NZ fur seal to sire pups. This confirmed the findings of Lancaster et al. (2007) on Macquarie Island of six pups which were assigned to non-territorial males. Two of these males were New Zealand fur seal hybrids which did not hold territories in the island breeding area.

We also found four resident males (two males that held territories for 29 and 46 days respectively and two non-territorial males) sired a total of eight pups born at neighbouring breeding areas (>100 m away, separated from the study site by non-breeding zones and boulder ledges). Although based on small sample size and scale, such observations are consistent with behaviours hypothesised to reduce inbreeding in systems that combine high reproductive skew and site fidelity: under pressures to limit inbreeding, females may seek copulations with other males than their immediate neighbours (Hoffman et al., 2007). By sampling pups outside our core study area, we found evidence of mixing between different areas of the colony (four resident males had pups in other parts of the 1 km long colony that same year), possibly due to females moving within the colony for mating. This hypothesis is backed up by our observations of identified females (n=3) regularly seen in one part of the colony without a pup during one breeding season, who pupped in another part of the colony the following season (Boren 2005). But Lancaster et al. (2007) found that the propensity of

fur seal females to mate extra-territorially was related to their reproductive status: nearly half of all females that mated outside territories did not give birth in the year of conception, and so were not induced to postpartum oestrus. In Antarctic fur seals, females observed without pups had a significantly lower chance of conceiving with a territorial male than females who did pup (Hoffman et al., 2003). This may reflect greater freedom in mate choice by females that are not constrained to a male's territory by their pup. However at Macquarie Island, females mating with non-territorial males have a high probability of mating with a heterospecific. Knowing that females at Macquarie Island actually discriminate against heterospecific males (Goldsworthy et al., 1999, Lancaster et al., 2007) it seems unlikely that extra-territorial pups are produced as a result of females preferentially. Instead, Lancaster et al. proposed that nulliparous females mate with heterospecific subordinate males for two reasons. They come into oestrus after the peak of the breeding season when territorial males have left or they are young and inexperienced and haul out to breed in areas away from main territorial areas. In the present study, it was not possible to identify which females gave birth in the year of conception and to relate non-territorial mating to a potentially late oestrus. As 90% of pupping takes place over six weeks at Ohau Point colony (Boren 2005), we approximate a similar duration for females postpartum oestrus. Only four out of our 38 study males were on site long enough (≥ 30 days) to mate with most receptive females. All other study males only managed to be present for a part of the season, further justifying the use of alternatives to costly territoriality.

Mating systems are typically conditional, and individuals are expected to exhibit the tactic that yields the greatest success relative to their status (genetics, size, age, experience, sex and stress hormones, health parameters, etc.) (Gross 1996). Smaller males are common at the periphery of breeding groups in most colonial pinnipeds (Bartholomew, 1953, Le Boeuf, 1974, Miller, 1975, Boness and James, 1979, McCann, 1980). Based on their phenotype, such males are considered as "inferior" ("marginal male effect" linked to female gregariousness, McLaren, 1967) and/or socially immature. After reaching sexual maturity, male pinnipeds need a few more years before reaching their full body size and enough experience to hold a territory ("delayed social maturation", e.g. Kiyota, 2005). Measuring the age of males is difficult (tooth extraction to count growth rings, which is highly invasive, Arnborn et al., 1992, or requires tagging at birth, Kiyota, 2005). In the grey seal, body mass rather than age determined the primary tactic employed (Lidgard et al., 2005). In the present

study, we could not age males, rather we estimated their size to see if it varied between mating tactic: the length of six territorial males in the current study (median=156.5 cm, range=128-168 cm) did not significantly differ from the length of nine territorial NZ fur seal males shot, aged and measured by Mattlin in 1978 (median=161 cm, range=140.3-181 cm, aged 9 to 14 years; Kruskal-Wallis $H=0.68$, $p=0.4$). Within the pool of males we measured, non-territorial were smaller than territorial males (although the difference between territorial and non-territorial residents was not significant, Table 4). A relatively smaller size is in agreement with the use of alternative tactics, theoretically characterising individuals that do not have an optimal territorial phenotype.

The potential for non-territorial males to successfully reproduce at the periphery of the group benefits these males who might be less successful than territorial males, but still “do better” than if they had no mates at all (Dawkins, 1980). For peripheral males that are socially immature, those able to increase their total lifetime reproductive success by producing a pup before becoming territorial could actually have a higher than average lifetime fitness.

Aside from individual status, ecological factors (e.g. site topography, resources for thermoregulation) are highly influential determinants of fur seal mating systems (Carey, 1989, Carey, 1991). They shape mating systems by influencing the aggregation of females and hence the potential for males to monopolize them (in mammals: Clutton-Brock and Harvey, 1978, in pinnipeds: Boness, 1991a, in fur seal: Bradshaw et al., 1999). Hoffmann et al. (2003) concluded there was no obvious alternative mating tactic in Bird Island Antarctic fur seals, despite the inability to assign parentage to 39% of the study pups and the recognition that the mothers of those pups most probably did not mate at the study beach. The Antarctic fur seal breeds in high densities on Bird Island, with an estimated density of 3.3 seals/m² – compared with 0.05 to 0.16 seal/m² in the NZ fur seal (Mattlin 1978, the present study, Bradshaw et al. 2000: summer range of mean pup density in twenty NZ fur colonies: 0.05-0.075 pup/m²). So the congestion in core breeding areas might prevent Antarctic fur seal males on Bird Island to use alternative tactics.

In addition to specific (in)tolerance to crowding (Gentry, 1975), local topography can explain such density differences in concert with colony developmental stage. In the current study, territory sizes were larger than previously reported for the NZ fur seal (Miller, 1974, Gentry,

1975, Miller, 1975, Mattlin, 1978), making it energetically impractical for territorial males to fully exclude subordinates. With the first pups born in 1990 and an exponential increase of 32% per annum (Boren et al., 2006), the Ohau Point colony still has considerable space for expansion (it is in the lower part of the density range for NZ fur seals breeding colonies, Bradshaw et al., 2000). This might partly explain the overall loose territorial setting, offering excellent pup rearing conditions (Boren et al. 2006) and allowing for non-territorial male tactics to be displayed (present study). The potential for alternative tactics in case of limited aggregation is illustrated in aquatically mating pinnipeds: in the water, females do not tend to aggregate and successful alternative male mating tactics were shown to co-exist in the bearded seal *Erignathus barbatus* (Van Parijs et al., 2003), the harbour seal *Phoca vitulina* (Boness et al., 2006) and the Weddell seal *Leptonychotes weddellii* (Harcourt et al., 2007).

Finally, recent studies have shown important inter-annual variations in the functioning and genetic structure of pinniped breeding colonies. In the grey seal, the level of polygyny is influenced by inter-annual climatic variations (Twiss et al., 2007); in the Antarctic fur seal, heterozygote/outbred males are more successful in low pup production years (Hoffman et al., 2004). The current study however shows that a single sampling season study can be highly cost-effective to document poorly studied behaviours, such as the potential for alternative mating tactics to yield males some success in a large colony of polygynous fur seals. Studies in a wide range of socio-ecological conditions (e.g. in both dense, mature populations and in more spread out, recolonising groups, Caudron et al., 2001) are essential complementary approaches for better understanding the subtle mechanisms underlying pinnipeds polygyny.

4.5 ACKNOWLEDGMENTS

We thank all the people who helped us in the field. Chris Muller and Tanguy de Tillesse provided invaluable help with crossbow sampling, with the kind assistance of Cathy Debier. Nicole Smolensky helped with the behavioural observations. Fieldwork was based at the Edward Percival Field Station, University of Canterbury, NZ. In 2002 and 2003, AC worked as a FNRS Scientific Collaborator (Belgium). Fieldwork and genetics work were covered by the FNRS (Bref Séjour grant, Crédit aux Chercheurs # 1.5.120.04.F), the Fonds Léopold III (Belgium), the post-doc Duesberg Fund (University of Liège, Belgium), the University of Canterbury (UC Masters scholarship and the Molecular Ecology Lab.) and a Minority

International Research Traineeships (USA) and associated funding. Samples were collected under the permit Per/10/2002/01, DOC, NZ; all experimental work was approved by the UC animal ethics and complies with the current NZ laws. Dr Paul Sunnucks (Monash) and Dr Jon Slate (Sheffield) made useful comment on an earlier version of this manuscript.

Chapter Five

Intra-sexual differences in testosterone levels and parasitism in a dimorphic polygynous mammal, the New Zealand fur seal

Negro S.S.¹, Caudron A.K.^{1,2}, Dubois M.³, Delahaut P.³, Gemmell N.J.¹

¹Molecular Ecology, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

²Unité de Biologie du Comportement, Institut de Zoologie, Université de Liège, 22 Quai Van Beneden, 4020 Liège, Belgique

³Laboratoire d'Hormonologie, Centre D'Economie Rurale, Rue du Point du Jour 8, 6900 Marloie, Belgique

5 Intra-sexual differences in testosterone levels and parasitism in a dimorphic polygynous mammal, the New Zealand fur seal

5.1 INTRODUCTION

Ecology and genetics have made important theoretical and empirical contributions to our understanding of life history. The principal life history traits are size at birth, growth rates, age and size at sexual maturity, number, size, and sex ratio of offspring, age- and size-specific reproductive investments, age- and size- specific mortality schedules and lifespan (Stearns, 1992). Although life history traits vary widely among populations, some combinations of traits are not observed in nature (Ricklefs and Wikelski, 2002). These traits are linked together by numerous trade-offs. For example, individuals in a population cannot simultaneously maximize fecundity and survival, or offspring size and offspring number (Roff, 2002). On a proximate level (immediate mechanisms responsible for a phenomenon), life history trade-offs are the consequences of restrictions in the availability of critical resources such as energy and nutrients, necessitating decisions on the differential allocation of resources to costly traits (Ganong, 1987, Zera and Harshman, 2001).

Increasing evidence implicates hormones as mediators of life history trade-offs (Rose and Bradley, 1998, Zera and Harshman, 2001, Knapp, 2004). Hormones transduce environmental cues and regulate transitions between life-cycle stages (e.g. metamorphosis, maturation, and reproduction) in which organisms face developmental constraints (Jacobs and Wingfield, 2000). Importantly, hormones can have pleiotropic and often antagonistic effects on morphological, physiological and behavioural characters (Sinervo and Svensson, 1998, Adkins-Regan, 2005).

Testosterone, the principal androgenic hormone, raises male mating success by promoting the development of secondary sexual characters. Testosterone promotes aggressive behaviours associated with courtship and reproduction in vertebrates (Alatalo et al., 1996, Wingfield et al., 2001, Garamszegi et al., 2005, McGlothlin et al., 2008), while decreasing fitness at the same time by impairing traits such as parental care and immune system

(Wingfield et al., 1990, Folstad and Karter, 1992). A schematic model of interactions between endocrine system, immune system, secondary sexual characters and mating success, and parasites is presented in Figure 1. Testosterone may have a negative impact on the immune system when beyond a threshold level. Wingfield et al. (1990) proposed a graphical representation of the 3-level model of testosterone in male birds where level A represents the non-breeding androgen baseline, level B represents the breeding androgen baseline induced by environmental cues, and level C represents the physiological testosterone maximum that an individual can achieve during intra- and inter-sexual interactions (Figure 2). Level B is necessary for spermatogenesis, expression of sexual behaviour, and the appearance of some secondary sexual characters, whereas level C is facultative and triggered by social stimuli or challenge during the breeding season. Level C corresponds to the frequency and intensity of territorial aggressiveness and female defence behaviour (Wingfield et al., 1990). The above models described for birds are supported by other studies in pinnipeds (Atkinson and Gilmartin, 1992, Bartsh et al., 1992, Lidgard et al., 2008).

5.1.1 Endocrine – secondary sexual characters interactions

Secondary sexual characters, such as various conspicuous ornaments, pheromones, behavioural displays, vocalizations, and aggressive behaviours, have dose dependent androgenic hormone profiles in mammalian, bird, lizard, and fish species (Schuurman, 1980, Noonan et al., 1991, Creel et al., 1993, Ramage-Healey and Bass, 2004, Hau, 2007, McGlothlin et al., 2008). Many studies have documented testosterone-induced behavioural changes, such as alteration of vocal patterns (Ramage-Healey and Bass, 2004), aggressiveness (Marler and Moore, 1988), and intensity and tenure of territorial behaviour (Bartsh et al., 1992). Zahavi's conditional handicap hypothesis (1975, 1977) predicts that males optimize the size of the ornament (secondary sexual character) depending on their physiological condition which impact on endocrine responsiveness. Thus males with poor physiological condition (poor endocrine response) have smaller optimal ornament sizes than males in good physiological condition as the cost of developing and maintaining an optimal ornament differ depending on the male's body condition.

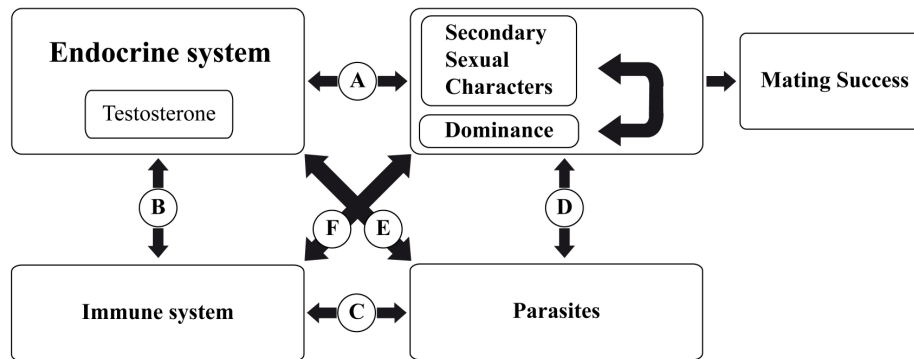


Figure 1: A simplified interaction model including endocrine, secondary sexual characters, immune and parasite components. Testosterone profiles have a positive effect on the expression of secondary sexual characters and dominance (A) which in turn may increase mating success, while impairing the immune function (B). Parasites interact with the immune system (C), may have a negative effect on the expression of secondary sexual traits and dominance (D), and may cause changes in testosterone profiles (E). The development of testosterone-dependent secondary sexual traits also co-occurs with a decline in immunocompetence (F). Folstad and Karter (1992) suggest a feedback loop, operating through the direct and indirect relationships connecting model components, and that links secondary sexual development to an individual's genetic resistance to parasites (modified from Folstad and Karter, 1992).

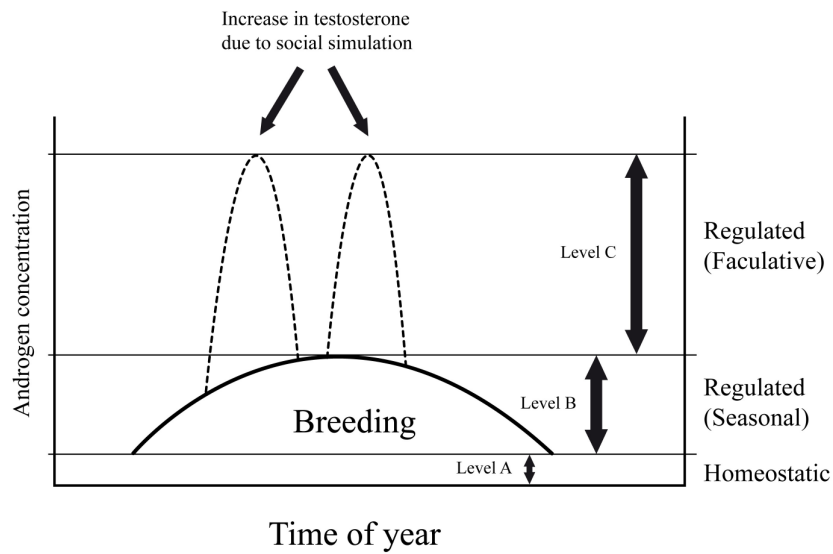


Figure 2: General patterns of circulating testosterone levels on male birds during the breeding season. level A represents the non-breeding androgen baseline, level B represents the breeding androgen baseline induced by environmental cues, and level C represents the physiological testosterone maximum that an individual can achieve during intra- and inter-sexual interactions (modified from Wingfield et al., 1990).

5.1.2 Endocrine – sexual and aggressive behavioural interactions

The challenge hypothesis predicts that increasing levels of testosterone generally increases the intensity of territorial aggression and mate guarding while decreasing parental care in birds (Wingfield et al., 1990). Several studies show the importance of sex steroids, such as testosterone, in regulating sexual and aggressive intra-sexual interactions. In experiments with free-living male lizards, Marler and Moore (1988) demonstrate an increase in aggressive intra-sexual interactions and male conspicuousness, and a decrease in survival among males possessing testosterone-implants compared to control males. Likewise, Dufty (1989) shows severe injuries in testosterone-implanted male brown-headed cowbirds due to the prolonged high testosterone levels and the intense aggressive intra-sexual interactions. Dufty (1989) proposes that the nature and importance of the different risks associated with high testosterone and increase sexual and aggressive intra-sexual behaviour vary with a species' mating system. Shuurman (1980) examine the effects of castration and subsequent testosterone administration on agonistic behaviour in adult male rats. Aggression decreased sharply after castration and increases after manipulating plasma testosterone concentration. Similar results are found in birds, rodents, and soay rams (Arnold, 1975, Lincoln, 1984, Hume and Wynne-Edwards, 2005, Soma, 2006). The experimental evidence above suggests the potential importance of endocrine control of aggressive behaviour which impacts on the dominance which in turn impacts on the ability to hold a territory and on mating success (Wingfield, 1984, Seely and Ronald, 1991, Bartsh et al., 1992, Ketterson et al., 1992, Creel et al., 1993, Moss et al., 1994, Alatalo et al., 1996).

An additional level of complexity is brought by some studies demonstrating that behaviour and social cues can also trigger the discharge of androgen and other hormones. Schuurman (1980) examine hormonal and behavioural changes after agonistic challenge experiments in rats. Rats behave significantly less aggressively and more defensively after suffering a serious defeat. The defeated rats show lowered testosterone and high corticosterone levels, which are associated with stress. Bartsh et al. (1992) examine territorial behaviour and breeding frequencies of male Weddell seals in relation to age, size, and steroid hormones. Male Weddell seals behave significantly less competitively after suffering a serious defeat. The defeated seals show a drop in testosterone levels accompanied by a transient rise in cortisol. Harding (1981) reviews social modulation of circulating hormone in vertebrate

males. The general hormonal trend of sexual interactions and exposure to female is an increase in luteinising hormone and testosterone levels.

5.1.3 Endocrine – immune system interactions

Testosterone interacts with the immune system at the level of both individual cells involved in the humoral- and cellular-mediated immunity and glands or tissues implicated in immune functions (reviewed in Grossman, 1985). Circulating testosterone levels above a specific threshold have been found to lower plasma immunoglobulin concentrations and specific immune responses in male mouse (Cohn, 1979). Casto et al. (2001) examine immune function in captive and wild monogamous male juncos that are treated with either testosterone-filled or empty implants. Prolonged elevation of testosterone impairs antibody production in captive males and cell-mediated immunity in wild males. On the other hand, Simkova et al. (2008) fail to show a suppressive effect of testosterone on immune function in cyprinid fish. Nevertheless a recent meta-analysis on bird, reptile, and mammal studies concluded that testosterone administration generally decreases immune function with different magnitudes of the testosterone effects within and between taxa (Roberts et al., 2004).

Testosterone might initiate its impact on immune system when above an individual's threshold level or at maximum physiological level (level C; Figure 2) and the range of testosterone effects on the immune function might be correlated to the length of exposure to high testosterone concentration. Monogamous and polygynous species generally differ in their breeding season profiles of circulating testosterone. Testosterone levels increase early in the breeding season of monogamous species when establishing territories and mate pairs and then declines. In contrast, testosterone levels remain high in polygynous species (Wingfield, 1984, Wingfield et al., 1990). Because of the prolonged high circulating testosterone profiles in polygynous species, immune function may be impaired at a higher degree compared to monogamous species.

The above meta-analysis and experimental evidence suggests that males with prolonged high testosterone concentrations may be more susceptible to disease or parasitism and ultimately, may reduce individual life span.

5.1.4 Endocrine – parasite interactions

The elevated testosterone levels required for the development and maintenance of secondary sexual characters expose a male to considerable risk to established or incident infectious agents (Folstad and Karter, 1992). Hoby et al. (2006) demonstrate a positive correlation between steroid hormone concentrations and parasite infections in free ranging chamois (*Rupicapra rupicapra rupicapra*) and reveal a male-biased parasitism. Recent field studies on Soay sheep (*Ovis aries*) support the hypothesis that sex differences in susceptibility to parasitism might incur sex differences in mortality. Male Soays exhibit a high degree of sexual size dimorphism (and consequently high levels of testosterone) and exhibit approximatively twice the female mortality rate (Clutton-Brock et al., 1997a, Coulson et al., 2001). Studies in a free-living lizard population (*Psammodromus algirus*), observe that male lizards implanted with testosterone possess larger patches of breeding colour, higher rates of aggressive intra-sexual interactions, and an increase in the number of ticks than control males. Ectoparasitism in male lizards negatively affects several haematological parameters and confers a higher mortality rate compared to control males, thus while there may be a reproductive benefit from testosterone implants, there is undoubtedly a cost to survival (Salvador et al., 1996). Decristophoris and co-workers (2007) examine the relationship between testosterone levels, dominance, body mass, age, and faecal egg counts. They demonstrate a positive correlation between testosterone and nematode egg count in a strongly sexually dimorphic and long-lived ungulate (*Capra ibex*). However, they find no relationship between the level of parasite burdens and any other tested variable. The mean faecal egg count found in their study (409 ± 266 eggs per gram of faeces) is considered to be indicative of moderate worm burdens. Some of the commonly found nematode species in the faeces of Alpine ibex are known to be associated with production losses and clinical disease in sheep (Brunsdon and Adam, 1975)

Interestingly, a meta-analysis on arthropods (crustaceans, ticks, insects) concludes that no positive correlation exists between males and parasite infections compared to females despite the greater intensity of sexual selection acting on males (Sheridan et al., 2000). Invertebrates have simple hormonal signalling pathways and the absence of testosterone and other steroid hormones may explain the absence of sex differences in parasite infections (Ruppert and Barnes, 1994).

5.1.5 Endocrine – nutrition interactions

Testosterone profiles can be regulated by energetic considerations. Testosterone has been shown to decrease sharply during food shortages (Wilson et al., 1979). Marler and Moore (1991) identify food limitation as an environmental factor that eliminates testosterone-implanted male mountain spiny lizards (*Sceloporus jarrovi*) deprived of supplementary food compared to testosterone-implanted males that were fed and males having empty implants. Testosterone-implanted males provided with supplementary food have their energy stores and survivorship increased compared to testosterone-implanted males deprived of supplementary food. These results support the hypothesis that the decreased level of survivorship of testosterone-implanted males may be the consequence of the high energetic costs related to increased territorial aggression (Marler and Moore, 1991).

5.1.6 Parasites – social status interactions

In an antihelminthic treatment experiment in red grouse, Fox and Hudson (2001) show that caecal nematodes decrease territorial behaviour. They use two approaches to investigate the influence of caecal nematodes on male aggressiveness. First, they compare territorial behaviour between a group of male treated with an anthelmintic which reduce parasitism and a group of control males with natural infection. Second, they compare the response of treated and untreated males to a conspecific territorial intruder using playback recordings. Their results show that treated males (with reduced parasite burdens) triumph significantly more territorial contests and more aggressive behaviour in response to the playback recordings compared to control males. Contrary to these results, Hinson et al. (2006) demonstrate that viral infection and reproductive success are independent of host social status in rats. In an experimental infection of male mice with *Taenia crassiceps*, Gourbal et al. (2002) show that infection is more likely to prevent adult male mice from becoming behaviourally dominant than to reverse existing dominant status. Parasites may prevent individuals attaining dominance or may impact on the tenure or level of dominance in some studies, in others parasites may have no effect of behaviour. Such contradictory results suggest that the parasites under investigation have different effects on the host physiology and behaviour. Variable power in analyses and stochasticity are other factors that can influence the variable

results among different studies. Negative correlations for parasite-mediated sexual selection may be explained by the fact that the particular parasite under consideration may not be the species that affects sexual selection in a particular host.

5.1.7 Parasite – secondary sexual characters

The correlation between the development of secondary sexual characteristics and parasite burden is controversial (Hausfater et al., 1990, Zuk et al., 1990). According to the immunocompetence handicap hypothesis (Folstad and Karter, 1992), the secondary sexual traits are assumed to be costly from an endocrine perspective and develop in response to circulating androgens, which on the other hand suppress the ability of an individual to raise an immune defence against parasites (Folstad and Karter, 1992). In this hypothesis, the signal for sexual selection is the extent of the secondary sexual development, the cost is the impairment of immune function, and genetic parasite resistance is what is indirectly being signalled.

5.1.8 Testosterone and costs of reproduction

In all animal taxa, costs are associated with reproduction which can reduce future fecundity and survival (Reznick, 1985). If viability costs do not exist in some form, "natural selection would favour a never-ending increase of fecundity in all populations since no counterbalancing selection pressures (costs) would cancel out the advantages of high fecundity" (Partridge and Sibly, 1991). High testosterone levels attract predators due to increased activity associated with sexual and aggressive behaviours and induce costs endured from intra-sexual dispute. Maintaining high testosterone plasma concentration is energetically expensive, which can increase mortality (Marler and Moore, 1988). Suppression of paternal care can also be a cost of high testosterone levels in species exhibiting parental care (Wingfield et al., 1990). Finally, testosterone has negative effects on aspects of immune function across vertebrates (Alatalo et al., 1996, Zuk, 1996, Sheridan et al., 2000, Casto et al., 2001, Nelson et al., 2002, Muehlenbein and Bribiescas, 2005, Hoby et al., 2006, Decristophoris et al., 2007, Martin et al., 2008).

Several theoretical attempts have been proposed towards explaining the deleterious effects of

high testosterone levels on the immune functions. The immunocompetence-handicap hypothesis, as an extension of Zahavi's (1975) handicap hypothesis and Hamilton and Zuk's (1982) predictions of the utilitarian function of secondary sexual characters, states that the expression of male secondary sexual characters, testosterone responsiveness, and consequently immunosuppression are self-regulated in response to parasite burden with constraint imposed by genetic resistance (Folstad and Karter, 1992). This hypothesis assumes that the secondary sexual traits are costly and more so to individuals of low than high quality. Individuals investing considerably in secondary sexual characters may increase their immediate reproductive success while impairing their future success due to the morbidity and possible mortality following immunosuppression (Folstad and Karter, 1992). Resource allocation among activities with competing demands may be the main cause of immunosuppression. A contradictory hypothesis is the testosterone immunoredistribution hypothesis, which states that leukocytes are temporarily directed to a different compartment of the immune system in response to testosterone and other steroids (Braude et al., 1999). Braude and co-workers (1999) propose three mechanisms to explain the testosterone immunoredistribution. Testosterone may directly bind to leukocyte or endothelium receptors and trigger migration to specific tissues. Alternatively, testosterone may enhance corticosteroid levels which, in turn, trigger migration to specific compartments. Finally, high testosterone and corticosteroid levels may be correlated when stress is associated with intraspecific competition and/or courtship.

In polygynous species, both males and females experience viability costs of breeding and reproduction, but to different degrees. Males incur greater viability costs due to increased somatic growth including secondary sexual characters and male-male competition compared to the viability costs experienced by females from mate choice and reproduction (Reznick, 1985, Pomiankowski, 1987). In polygynous mammals, males compete for mates either by directly preventing other males from acquiring females (female or harem defence polygyny) or by excluding other males from an essential resource required by females (resource defence polygyny) (Berta et al., 2006). The greater costs experienced by polygynous males include injuries from physical conflicts incurred while acquiring and maintaining absolute access to females, a decrease of nutritional intake and subsequently decreased growth and depletion of body reserves, and increased susceptibility to pathogens to a level which may affect survivorship (Deutsch et al., 1994).

New Zealand fur seals are polygamous and annual colonial breeders. The breeding season begins when adult males come ashore and establish territories in the austral spring. Females haul out from mid-November to late December to give birth (Mattlin, 1978, Goldsworthy and Shaughnessy, 1994, Boren, 2005). Females enter oestrus approximately one week after parturition followed by alternation between foraging at sea and pup-nursing onshore. The breeding season ends when all females terminate their oestrous cycle and initiate their foraging cycles in February (Goldsworthy and Shaughnessy, 1994).

The life history traits of New Zealand fur seals and other pinniped species are linked by several fundamental characteristics related to the evolution of their socio-sexual behaviour. They are marine feeders, but come onshore for parturition and postnatal pup care (Bartholomew, 1970). Reproduction in pinnipeds is characterized by highly synchronized pupping and mating, except for the tropical and subtropical species such as monk seals which have a lower degree of synchrony (Fritz and Trillmich, 1984), in addition to a delayed implantation of the blastocyst (Atkinson, 1997).

The degree of polygyny is determined by the average number of females defended by a territorial male. In pinnipeds the polygyny ranges from extreme (northern fur seal and elephant seal) where one male may mate with 16-100 females, to moderate (New Zealand sea lion) where one male may mate with 5-15 females. New Zealand fur seals display moderate polygyny where one male may mate with 5-8 females (pers. obs.).

Although an abundant literature has described the impact of sexual dimorphism on morphology, physiology, and parasitic infections, little is known about the intra-sexual differences in physiology and the impacts of parasite infections on the reproductive success of male mammals of different social status, employing different mating tactics. This study examined the viability costs of sexual selection between territorial and non-territorial males related to their relative reproductive success. First, we collected behavioural observations on individually identifiable males which allowed us to assign a mating tactic to the study males. We measured testosterone levels of males displaying territorial and non-territorial mating tactics using non-invasive sampling methods. We examined the parasitic species diversity in relation to the social status of the known study males. Finally, we correlate the above results

with the relative reproductive success of the territorial and subordinate males mating tactics found in a parallel study using the same focal area (Chapter 4). This study shed insight on the micro-evolutionary processes underlying polygynous mating strategy displayed by a dimorphic polygynous mammal, the New Zealand fur seal.

5.2 MATERIAL AND METHODS

5.2.1 Study site

The Ohau Point seal breeding colony (ca. 2,200 individuals; Boren, 2005), located 26km north of the Kaikoura township, New Zealand (42°25'0"S, 173°40'60"E), is a narrow rockery colony (50-100m wide) 1km long running along the Pacific Ocean coast and situated close to their foraging ground. Our study area was limited to the central portion of the colony (50-90m wide and 120m long, backed by a cliff on the west). In the 19th century, New Zealand fur seals were hunted nearly to extinction by the fur trade, but are now protected under the New Zealand's Marine Mammals Protection Act (1978). Breeding New Zealand fur seals have recently re-colonized the Kaikoura region (Report by The New Zealand Department of Conservation). They recovered slowly throughout their pre-exploitation range from 1990 until 1999 (Boren, 2001), but sites on the Kaikoura coast, particularly the Ohau Point breeding colony, have now entered a stage of exponential population growth (Boren et al., 2006).

5.2.2 Behavioural sampling

We undertook 223 hours of field observations spread from mid-November 2004 to early January 2005 coinciding with the arrival of pregnant females and covering the period of highest intra- and inter-sexual interactions. The total number of adult males that spent more than 3 hour during the study period was ca. 70. Fifty-two of 70 males were readily identifiable by natural marking (e.g. body scars, flipper irregularities) or artificial marking (i.e. cattle ear tag or sheep tag from previous studies) and these were selected for further study.

We quantified all interactions involving focal males by "all occurrences sampling" (Altmann,

1974). We categorized these interactions as described Chapter 4 with the following modifications. The intra-sexual interactions involved walking towards, following, chasing, attacking, lunging at, and fighting with another male. We attributed vocalizations to either addressed (specific) or non-addressed (unspecific) male target. For each intra-sexual interaction, the study males were classified as aggressive (the male initiating the interaction and/or displacing or triumphing over the other male) or submissive (the target male and/or displaced male). The inter-sexual interactions involved walking towards, withholding, attempting to copulate and successful copulation, accepting or tolerating female advances (soliciting, mounting on the male's back, and biting the male's neck) and vocalizing to a female.

We used six out of seven variables described in detail in Chapter 4, to quantify the breeding behaviour of the study males : 1) the mean frequency of aggressive intra-sexual interactions, 2) the mean frequency of submissive intra-sexual interactions, 3) the mean frequency of inter-sexual interactions, 4) the mean frequency of male vocalization to female, 5) the mean frequency of male vocalization to addressed or non-addressed male target, and 6) defence or non-defence of territory containing hosting females.

5.2.3 *Testosterone assays*

We used a non-invasive sampling approach to collect faecal (n=10) and urine (n=7) samples from different known males (2004-05 breeding season), for which extensive behavioural data were available, to minimize disturbance of the seal mating behaviour as this species is sensitive to human intrusion and even more to capture (Boren et al., 2002). We also collected one faecal sample and several urine samples from females that were used as controls. The androgen concentrations in urine and faeces integrate changes in circulating blood androgen concentrations over many hours of metabolism. Thus, androgen measures in excretion products more accurately reflect the long-term differences in circulating androgens among animals (Solomon et al., 1990, Withers, 1992, Randall et al., 2000). Contrary to blood sampling, urine and faecal sampling is compatible with behavioural study of breeding male pinnipeds where human effects on behavioural changes of the study individuals are minimized. The sample collections spread from mid-November 2004 to early January 2005 from the periphery or outside the male territories to minimize disturbance. Territorial males generally urinated or defecated after chasing a subordinate male or on their way back to the

centre of their territory.

Urine and faecal steroid represents a mixture of several steroids with different polarities and needs to undergo different steps of purification (Goymann, 2005, Möstl et al., 2005, Palme et al., 2005). We performed a preliminary extraction step (modified from Möstl et al., 2005, Palme, 2005) followed by a clean-up step using immunoaffinity chromatography columns (IAC) and reverse-phase high performance liquid chromatography (HPLC) prior the testosterone radioimmunoassay (RIA).

Urine steroid extraction:

We aliquoted 1ml of urine per urine sample and added 1.5ml of 0.2M acetate buffer (pH 4.6) and 10µl of *Helix pomatia* juice (Boehringer, Mannheim, Germany). We adjusted the pH of the solution to 7 with 10ml PBS buffer after incubation overnight at 37°C. We centrifuged the solution prior the clean-up step.

Faecal steroid extraction:

We aliquoted 0.3g of dry faeces per faecal sample, added 6.4ml of methanol and vigorously vortexed the solution for 1h prior to overnight incubation at 4°C. We added 1.6ml of water and 0.1ml HCl 1N, and vortexed for 30min. After centrifugation and decantation, we added 0.1ml NaOH 1N and 5ml light petroleum to the pellet and vortexed the solution for 15min. We centrifuged the preparation at 2700xg for 15min and then removed the light petroleum and solid material using a mild vacuum. Finally, prior the clean-up step, we diluted the solution with 20ml of water and 20ml of PBS buffer followed by centrifugation.

Clean-up step on immunoaffinity columns (IAC):

We purified the urine and faecal extracts using IAC containing polyclonal antibodies raised against testosterone (binding capacity to testosterone 250ng) (CER Groupe, Marloie, Belgium). We brought the columns to ambient temperature and removed the buffer solution. We added 5ml PBS buffer and rinse the columns with 10ml water before loading the urine and faecal extracts onto the columns. We rinsed the columns with 10ml water and 10ml methanol/water (20/80, v/v). Finally, the columns were eluted with 3ml methanol/water (80/20, v/v). We evaporated the IAC extract residues and then dissolved the pellet in 200µl acetonitril/water (40/60, v/v) prior to HPLC.

Reverse-phase high performance liquid chromatography (HPLC):

We performed an additional purification step from the IAC extract residues using HPLC. The high performance liquid chromatography system includes three Gilson pumps (Models 305 and 306) controlled by a AST Bravo LC4/33 computer: (1) Gilson 232XL automatic injector; (2) Gilson 118 monochromator UV detector set at 254nm; (3) Gilson 202 fraction collector (Analisis, Namur, Belgium). We separated testosterone from other steroid hormones on a reverse-phase ODS C18-column (150 x 4.6 mm id, 5 µm film thickness) (Beckman Coulter, Fullerton, USA) using an isocratic solvent system of acetonitril/water (40/60, v/v) at a flow rate of 1.5ml per minute. We calibrated the column using ³H-testosterone and collected the testosterone fraction between 6.0-6.6min.

Testosterone radioimmunoassay and validation:

We evaporated the testosterone fraction and added 1ml RIA buffer. We used two aliquots of 50µl for RIA and 300µl for calculating the extraction efficiency. We measured urine and faecal testosterone using a Testosterone RIA Kit (CERGroupe, Marloie, Belgium). The kit was based on the ability of polyclonal antibodies to bind either testosterone or labelled ³H-Testosterone. Testosterone molecules competed with the labelled ³H-Testosterone for the antibody sites. To determine the testosterone concentration, we used an assay system including an antiserum raised against testosterone-3 CMO-BSA that cross-reacted 32 % with dihydrotestosterone and less than 6 % with 5α-androstan 3,17 diols.

The intra-assay coefficients of variation in urine and faeces were 20 % and 25 %, respectively. The limit of detection was 0.2 ng/ml in urine and 0.3 ng/ml in faeces. Extraction efficiency was determined by the recovery of ³H-Testosterone (6,000 cpm) added to the samples prior to extraction and was 33±5% (mean±SD) for urine and 18±3% for faeces.

5.2.4 Parasitic load and species richness in faecal samples

We used the same faecal samples (n=10) collected for the testosterone assays in addition to faecal samples (n=3) collected from other known males to estimate the parasitic load and species diversity (trematodes, cestodes, nematodes, acanthocephalans) in sampled males. A modified McMaster quantitative method was used to estimate the number of parasite eggs per gram of faeces (Thienpont et al., 1979). We added 56ml of saturated NaCl (density of

1200 kg.m⁻³ at 21°C) to 4g of faecal material. After mixing, we filtered the solution through a 150µm sieve. After mixing, we took a few ml of the suspension and filled in the two chambers (10x10mm) of the McMaster slide and let the suspension rest for 5min before observing the slide with a microscope at 10x10 magnification. The egg count for each parasitic species was multiplied by 25 to determine the number of eggs per gram of faeces.

5.2.5 *Statistical analyses*

Parametric statistical methods (Student's and Welch's Two Sample *t*-tests) were used except when the assumption of normality of the data distribution was violated. In this situation, nonparametric tests (Spearman rank correlations) were used. For identifying male behavioural classes, we used an agglomerative hierarchical clustering based on similarities in their behavioural profile using Ward's linkage of Euclidean distances (Johnson, 1967, StatSoft, 2007). The probability level used for significance was $\alpha=0.05$. The standard error (S.E) is shown with the means. Statistical analyses were performed in STATISTICA v8 (StatSoft, 2007) and R v1.7.1 (R Copyright, 2003).

5.3 RESULTS

5.3.1 *Behavioural sampling*

Approximately 70 adult males spent more than 1 hour at our study site during the study period. Three out of 52 identifiable breeding males spent less than three hours and were excluded from the analyses. We used six variables to describe the breeding behaviours of the 49 remaining males. The mean frequency of aggressive intra-sexual interactions per male ranged from 0 to 1.80 per hour. The mean frequency of submissive intra-sexual interactions per male ranged from 0 to 0.67 per hour. The mean frequency of dominant male vocalizations to addressed (specific) or non addressed (unspecific) male target ranged from 0 to 3.04 per hour. The mean frequency of inter-sexual interactions per male ranged from 0 to 1.86 per hour. The mean frequency of male vocalization to females ranged from 0 to 1.07 per hour.

A large proportion of study males (69%, n=32) behaved in a subordinate way (moving away

to avoid any intra-sexual interactions), while a smaller proportion (31%, $n=17$) defended a territory containing hauled-out females. We identified 12 topographically demarcated territories defended by territorial males.

We used an agglomerative hierarchical clustering approach to sort the study males into objective classes based on their use of similar male behaviours. The cluster analysis identified two classes, the dominant territorial profile and the subordinate non-territorial profile (Figure 3). Territorial males showed a higher mean frequency of aggressive intra-sexual interactions (0.63 ± 0.09 per hour in territorial males versus 0.11 ± 0.03 per hour in non-territorial males; Student's t -test, $t=5.99$, $df=47$, $p<0.001$), male-female interactions (1.06 ± 0.1 per hour in territorial males versus 0.30 ± 0.07 per hour in non-territorial males; $t=6.41$, $df=47$, $p<0.001$) and vocal pattern to addressed and non-addressed male target (0.53 ± 0.14 per hour in territorial males versus 0.09 ± 0.05 per hour in non-territorial males; $t=3.29$, $df=47$, $p=0.002$) compared to the non-territorial male. Territorial males also showed a lower frequency of submissive intra-sexual interactions (0.07 ± 0.01 per hour in territorial males versus 0.20 ± 0.03 per hour in non-territorial males; $t=-3.55$, $df=47$, $p<0.001$). Finally, the territorial males vocalized to females at a higher rate compared to non-territorial males (0.34 ± 0.07 per hour in territorial males versus 0.04 ± 0.02 per hour in non-territorial males; $t=4.71$, $df=47$, $p<0.001$).

In Chapter 4, we presented evidence that alternative mating tactics exist among males in the Ohau Point study area and we described in detail the range of male behaviours and identified three main classes (i.e. territorial, non-territorial resident, and transient males). In this study, we observed two classes (territorial and subordinate non-territorial) because we used simplified behavioural data collection and fewer observers than for Chapter 4.

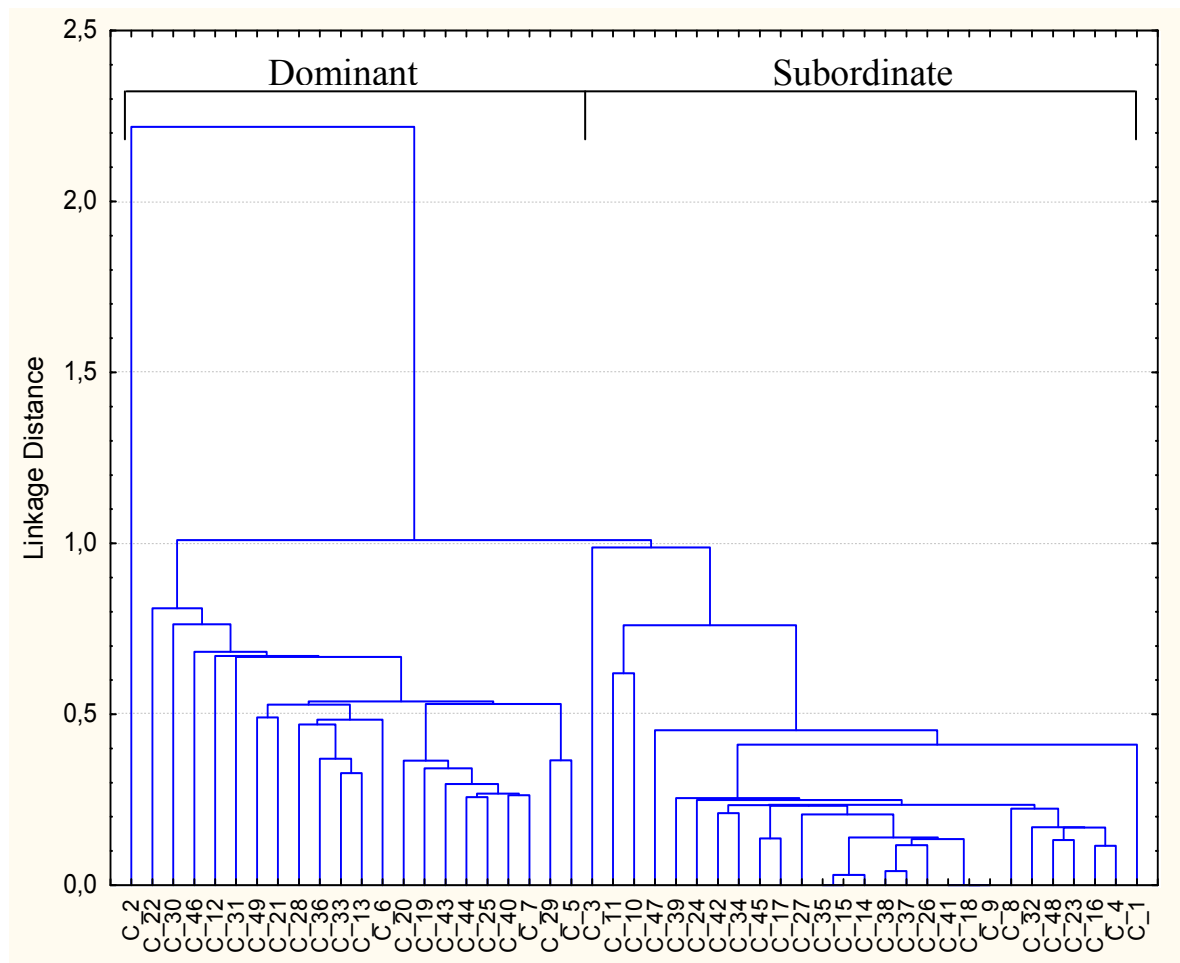


Figure 3: Hierarchical clustering of male behavioural profiles using Ward's linkage of Euclidean distances. Each profile represents an individual. The cluster analysis suggested two classes corresponding to the dominant territorial class and the subordinate non-territorial class. Territorial males had a significantly higher frequency of aggressive intra-sexual and inter-sexual interactions. In addition, territorial had increased rates of vocalization to females and to both addressed and non-addressed male targets, while having a lower frequency of submissive intra-sexual interactions.

5.3.2 Testosterone assays

We measured testosterone concentrations in urine (n=7) and faecal (n=10) samples from study males representing both the territorial and non-territorial behavioural categories. Levels of testosterone in the males using the primary mating tactic (faeces, n=4, mean \pm SE 55.21 \pm 9.95 ng/g dry faeces; urine, n=2, 7.43 \pm 3.54 g/ml) were significantly higher (for the faecal samples) than the levels of testosterone observed in the non-territorial males (faeces, n=6, 5.17 \pm 1.85 ng/g dry faeces; urine, n=5, 1.43 \pm 0.32 g/ml) (Figure 4). The number of urine samples tested was too small to infer any conclusive results between male groups (Figure 4). The significance of the difference between the mean of T and NT males has been tested using a randomisation test. We prepared vector for 1000 differences of randomised data (from real data). The 0.975 quantile (37.97) and normal 0.975 quantile (34.85) using the faecal testosterone were smaller than the difference between mean of T and NT males in real data (50.04). The 0.975 quantile (6.98) using the urine testosterone was about one unit greater than the difference between T and NT in real data (5.99), and the normal 0.975 quantile (5.94) approximated the difference between T and NT in real data (5.99). Levels of faecal testosterone were positively correlated with the mean frequencies of intra- and inter-sexual interactions (Spearman rank correlation, $r_{s10}=0.79$, $p=0.006$), the mean frequency of vocalization to females, and vocalizations to addressed and non-addressed male targets ($r_{s10}=0.74$, $p=0.014$). We found no conclusive correlation between urine testosterone and intra- and inter-sexual interaction and pattern of vocalization (Figure 4).

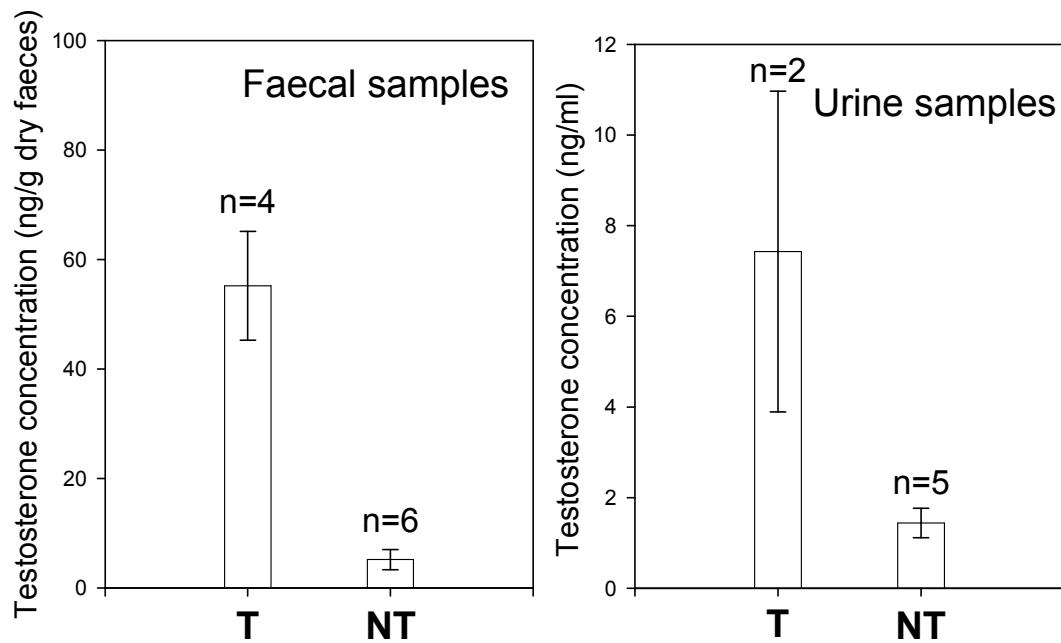


Figure 4: Testosterone measurement in faecal and urine samples from territorial males (T) and non-territorial males (NT). Faecal testosterone levels in territorial and non-territorial males are significantly different (Welch two sample *t*-test, $t=4.94$, $df=3.21$, $p<0.01$). Urine testosterone levels in territorial males were high compared to urine testosterone levels in non-territorial males, but the difference was not significant (*t*-test, $t=1.69$, $df=1.02$, $p<0.33$) because of the low number of territorial male samples ($n=2$). Female testosterone levels in faeces (2.1ng/g dry faeces) and urine (0.167ng/ml) were used as controls.

5.3.3 Parasitic load and species richness in faecal samples

A total of 14 faecal samples from known individual males were screened for parasite species (trematodes, cestodes, nematodes, acanthocephalans). We had testosterone measurements for 10 faecal samples out of 12 different individual males for which we had parasite loads, and we had duplicate faecal samples from two known individual males.. We found 4-8 and 1-4 parasite species per territorial male and non-territorial male respectively. The parasite species identified were: trematodes; *Synthesium* sp., *Zalophotrema* sp., *Pricetrema zalophi*; cestodes; *Diphylobothrium* sp.; nematodes; *Ascaris* sp., *Anisakis* sp., *Ostoststrongylus* sp., *Contracaecum* sp.; acanthocephalans; *Bolbosoma* sp., *Corynosoma* sp.; and two unidentified parasite species. The parasite loads for each individual male are detailed in Table 1. Two out of five territorial males had impressive numbers of eggs per gram of faeces (T2 and T3 in Table 1) The original sites of parasite infection and the pathogenic characteristics of the above parasite species extracted from male New Zealand fur seal faeces are summarized in Table 2.

Territorial males were more often parasitized by different species compared to non-territorial males (Welch two sample *t*-test, $t=4.85$, $df=6.62$, $p<0.002$). The mean number of parasitic species per individual was 6.2 ± 0.66 for the territorial males and 2.5 ± 0.38 for the non-territorial males (Figure 5). The parasitic species richness increased as a function of testosterone levels measured in the faecal samples from territorial (T) and non-territorial (NT) males (Figure 6). In contrast, this relationship is negative within male groups (regression; T, $R_{sq} = 0.387$, $y = 9.48 - 0.06x$; NT, $R_{sq} = 0.577$, $y = 3.68 - 0.16x$).

Table 1: Parasite load (eggs per gram of faeces) and species diversity found in each individual male (n= T, territorial male, NT, non-territorial male). The egg counts per gram of faeces in a female sample were used as control.

	Control	T1	T2	T3	T4	T5	NT1	NT2	NT3	NT4	NT5	NT6	NT7	Mean T	Mean NT
Cestode															
Diphyllobothrium	500	125	125	250	-	500	100	-	75	125	250	500	-	250±88	210±79
Nematode															
Ascaris	100	125	125	500	-	250	250	-	125	-	-	-	125	250±88	166±42
Anisakis	-	-	-	275	125	250	-	-	-	-	-	-	-	217±46	-
Contracaecum	-	-	1575	2625	-	250	-	-	100	100	-	-	-	1483±1190	100
Ostoststrongylus	-	125	-	-	-	125	-	-	-	-	125	75	-	125±0	100±25
Acanthocephalans															
Bolbosoma	-	-	1550	-	125	375	-	250	-	375	250	375	-	683±439	312±36
Corynosoma	-	-	50	-	-	-	-	-	-	-	-	-	-	50	-
Trematode															
Synthesium	-	-	125	2975	-	500	-	-	-	-	-	-	-	1200±894	-
Pricetrema	-	-	-	50	-	-	-	-	-	100	-	-	-	50	100
Zalophotrema	-	-	-	125	-	-	-	-	-	-	-	-	-	125	-
UID* 1	-	100	125	-	125	-	-	-	-	-	-	-	-	117±8	-
UID* 2	-	125	75	-	75	-	-	-	-	-	-	-	-	92±17	-

*UID: unidentified

Table 2: Parasite species found in faecal material of male New Zealand fur seals, original body part of parasite infection, and clinical signs reported in otariids and phocids.

Parasite	Original site of infection	Clinical signs in otariids and phocids	Notes
Cestodes			
• <i>Dipyllobothrium</i> spp.	GI	Pathogenic signs vary widely between hosts (Lauckner, 1985, Ionita et al., 2008)	Burdens may be high; infection vary seasonally
Nematodes			
• <i>Ascaris</i> spp.	GI	Gastritis, gastric ulceration, enteritis, diarrhea, dehydration, anemia, and gastric perforation (Young and Lowe, 1969, Ridgway et al., 1975, Lauckner, 1985)	Burden may be high with no apparent clinical signs
• <i>Anisakis</i> spp.	GI	As above	As above
• <i>Contracaecum</i> spp.	GI	As above; Peritonitis and death induced by perforated ulcers in the proximal duodenum in California sea lions (Fletcher et al., 1998)	As above
• <i>Ostostromylus</i> spp.	RS	Vary widely between hosts; anorexia, depression, dehydration, neutrophilia, disseminated intravascular coagulation, death in elephant seals (Gulland et al., 1997)	-
Acanthocephala			
• <i>Bolbosoma</i> spp.	GI	Death reported in Northern fur seal (Ionita et al., 2008)	-
• <i>Corynosoma</i> spp.	GI	As above	-
Trematodes			
• <i>Synthesium</i> spp.	GI	ND	-
• <i>Pricetrema zalophi</i>	GI	Colitis in infected elephant seals (Dailey, 2001)	Burdens may be high with no apparent clinical signs
• <i>Zalophotrema</i> spp.	Liver	Meningoencephalitis induced by aberrant trematode migration in California sea lions (Fauquier et al., 2004)	-

GI gastrointestinal

RS respiratory system

ND no data available

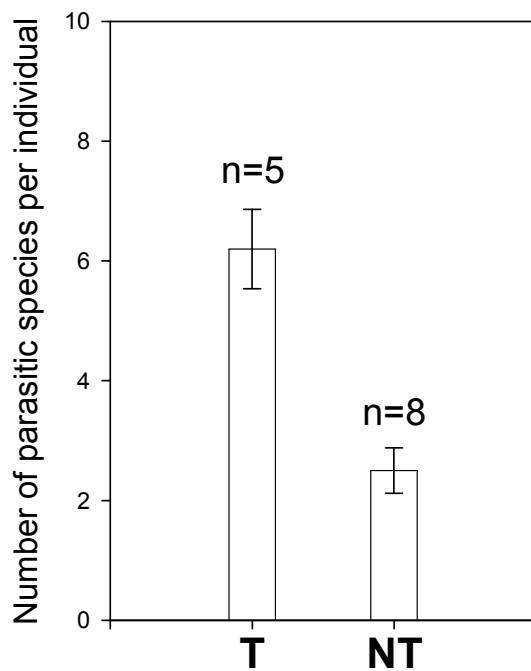


Figure 5: Average number of parasitic species per dominant territorial male (D) and subordinate non-territorial male (S). Parasitic species richness in the dominant males was significantly higher than that found in the subordinate males.

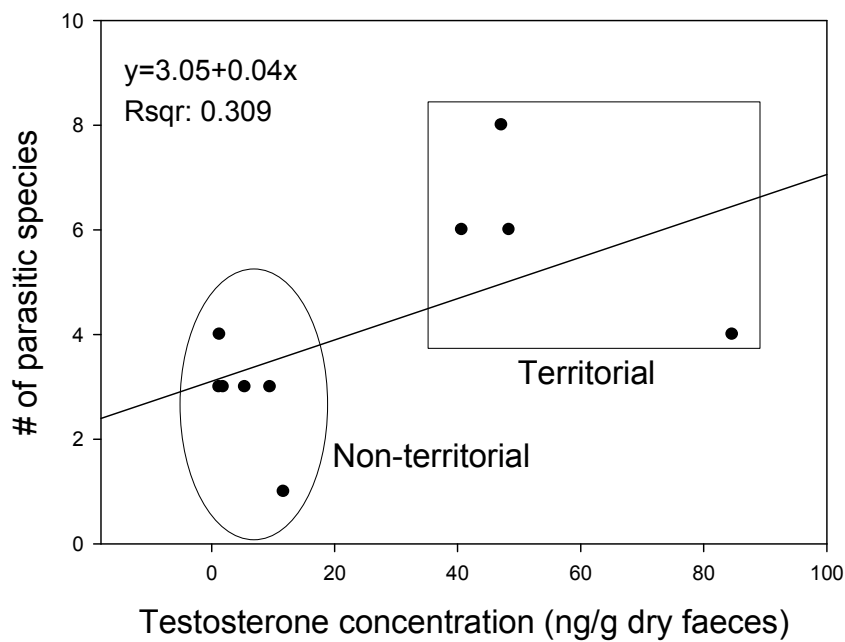


Figure 6: Parasitic species richness as a function of testosterone levels measured in the faecal samples of the subordinate non-territorial (circle) and territorial (square) males.

5.4 DISCUSSION

The pattern of allocation of resources to growth, maintenance, and reproduction defines an individual life history throughout its lifetime. Testosterone, the principal androgen, is the prominent candidate involved in the mechanism for mediating life history trade-offs across vertebrate taxa (Folstad and Karter, 1992, Ketterson and Nolan Jr, 1992, Muehlenbein and Bribiescas, 2005). On one hand, testosterone has been shown to promote courtship, reproductive behaviours, territorial aggression, secondary sexual traits, and sperm production, which may increase male reproductive success (Wingfield et al., 1990, reviewed in Hau, 2007). On the other hand, testosterone has been shown to impair immune function and parental care, and ultimately decrease fitness (Folstad and Karter, 1992, Ketterson et al., 1992, Ots and Horak, 1996). Testosterone immunosuppression may be the result of resource allocation among activities with competing demands e.g. resources necessary for the maintenance of the immune functions may be reallocated to the production of costly secondary sexual characters, which must have high priority in order to increase mating success (Folstad and Karter, 1992).

The New Zealand fur seal is a dimorphic polygynous mammal and territorial males can reach three times the adult female size (Miller, 1971, Carey, 1989). Testosterone levels in territorial New Zealand fur seal males were significantly higher than the testosterone levels in the non-territorial males (Figure 4). This testosterone pattern is generally encountered in territorial and high-ranking individuals across vertebrate taxa (Sapolsky, 1986, Wingfield et al., 1990, Bartsh et al., 1992, Gould and Ziegler, 2007, Shargal et al., 2008).

Body size is an important facet of social rank in many mammals and particularly so in polygynous species where it directly influences the outcome of intra-sexual conflicts over access to females and consequently male reproductive success (Clutton-Brock et al., 1988, Le Boeuf and Reiter, 1988, Desjardins et al., 2008). The degree of expression of secondary sexual characters was higher in territorial males as expressed by a higher mean frequency of vocalization to females and to male targets and undirected targets compared to non-territorial males. Kunc and Wolf (2008) have demonstrated that vocal displays are important in sexual selection through both male-male competition and female choice in male Galápagos sea lion (*Zalophus wollebaeki*). In fish, birds, and mammals vocal displays are used to establish and

maintain a territory and have been proposed to be an indicator of male quality (Slater, 1981, Reby et al., 1999, Ramage-Healey and Bass, 2004, Kunc and Wolf, 2008). Evidence in amphibians and birds suggests that vocalizations result in relatively low energetic costs (Grafe and Thein, 2001, Thomas, 2002, Ward and Slater, 2005, Waas, 2006). The main costs for a territorial otariid male might be related to the permanent presence in its territory (estimated to 46 days in our study area; Chapter 4), the costs endured from male-male dispute, and the depleted energy reserves from fasting during territory tenure rather than the vocal display itself. In the New Zealand fur seal, the occurrence of serious fights is very low (Carey, 1989; pers. obs.), which suggests that vocalizations regulate territorial conflict. The intra- and inter-sexual functions of vocalization might be to avoid costly confrontations since males might use vocal and visual cues to assess each other's current fighting ability and physical condition, while allowing females to assess a male's quality.

Territorial New Zealand fur seal males showed more aggressive and sexual (dominant) behaviours compared to the non-territorial males and this result therefore meets with observations from earlier studies. Demas et al. (2006), in a synthesis derived from rodent, avian, and primate studies, conclude that testosterone boosts reproductive effort by heightening sexual and aggressive behaviours, which in turn may provide advantages to male mating success. Direct manipulation of testosterone levels in birds shows that the behavioural and physiological effects of testosterone can influence the major components of fitness, particularly male reproductive success (Wingfield, 1984, Ketterson et al., 1992). McGlothlin et al. (2008) demonstrated positive correlations between the magnitude of testosterone concentration and the size of plumage ornament, which is an important determinant for female choice and male-male competition, in a population of dark-eyed juncos (*Junco hyemalis*). Other studies in mammals and birds show similar positive effects of heightened testosterone on vocal displays and reproductive success (Bartsh et al., 1992, Ketterson et al., 1992, Alatalo et al., 1996).

Chapter 4 has shown that territorial male New Zealand fur seals were larger and had higher reproductive success compared to non-territorial males in the same study area, which implies that higher testosterone is linked to dominance documented by higher body mass and higher reproductive success. On the other hand, we found a positive relationship between male dominance, testosterone levels and parasitic loads/species diversity among territorial and

non-territorial males (Figure 4, 5, and 6). In contrast, we found a negative relationship between testosterone levels and parasite species diversity within the territorial and non-territorial classes (Figure 6). All parasite species are not equally virulent, so that greater numbers of parasite species in one host than in another host do not necessarily equate with a greater impact of parasitism. In addition, the virulence of any particular parasite species is often dependent on the intensity of infection (Table 2). However, the high parasite egg counts in some territorial males (T2 and T3) may well imply that there is a positive association between dominance, heightened testosterone levels, parasitic infection, and potentially mortality (Table 1 and 2).

The New Zealand fur seal has undoubtedly been exposed to many parasites over an evolutionary timescale. It is conceivable then that NZ fur seal males may have evolved a mechanism that enables them to present evidence of their parasite resistance to females, such as the elaborate plumage displays of some birds (Hamilton and Zuk, 1982, Folstad and Karter, 1992). If such a signal has evolved then we expect a positive relationship between secondary sexual characters (e.g. body size, vocalization, territorial behaviour, etc) and parasite resistance in the NZ fur seal male population (Table 1, Figure 5 and 6). However, we observed the opposite trend within male groups (T and NT; Figure 6) in our study, with territorial males exhibiting higher testosterone levels and parasite burdens than non-territorial males. This observation is at odds with the idea that males with heavy parasite burdens might be unhealthy and unable to spare resources for investment in secondary sexual characters, which would further support the negative relationship between secondary sexual characters and parasite load over a short term, phenotypic, timescale. However, another possibility is that the secondary sexual characters, parasite loads and testosterone are interdependent with males expressing high levels of testosterone to generate strong secondary sexual characters causing themselves to become immunosuppressed and susceptible to parasitism. Under such a scenario we expect a positive relationship between testosterone levels and parasitism on a species-evolutionary timescale. On a short term, phenotypic, timescale, we might expect the opposite relationship.

Components of our results are not without precedence. A comparative study in mammals indicates that parasites contribute to the association between sexual size dimorphism (SSD) and male-biased mortality (Moore and Wilson, 2002). Sex-biased parasitism is positively

correlated with the degree of sexual selection, which is in turn related to the degree of SSD, and with male-biased mortality across mammals (see also Promislow, 1992). Other studies have shown positive correlations between SSD, the degree of polygyny and the degree of sexual selection in mammals (Clutton-Brock et al., 1977, Alexander et al., 1979). Testosterone and other androgens may be important proximate factors in regulating the relationship between SSD and male-biased mortality, with experiments in lizards and birds showing higher mortality rates (from high energetic costs of aggressive behaviour and development of secondary sexual characters) in males implanted with testosterone compared to non-implanted males (Marler and Moore, 1988, Dufty, 1989, Moss et al., 1994).

An abundant literature on sex differences in morphology, physiology, and parasite infections is available, but integrated studies on intra-sexual differences in behaviour, male displays, morphology, physiology, and parasite infections associated to the reproductive success of the different mating tactics in mammalian species are largely unexplored. A few studies examining the wild population of Soay sheep on the remote island of St Kilda have taken an integrated approach to this problem. The interactions between reproductive success, mating tactics, testosterone, and morphology have been examined (Stevenson and Bancroft, 1995, Preston et al., 2003), while Coltman and collaborators (2001) have looked at parasite burdens and their relationship to fitness in Soay sheep. This study showed positive correlations between dominance, aggressive and sexual behaviour, testosterone levels, and potentially parasite loads. In addition a joint study at the same study site showed a positive relationship between male dominance, male body size, and reproductive success (Chapter 4; four territorial males from chapter 4 could be sampled for chapter 5). From the immunocompetence handicap hypothesis point of view, the interpretation of these results would be that the territorial males are of such high quality overall that they can display and attract females despite higher parasite infection due to immunosuppression (Folstad and Karter, 1992). Female mate choice may be under indirect selection. The benefits to choosy females may either be that they produce more attractive offspring or that the offspring produced, which would have inherited the genes for disease and parasite resistance or other characteristics, are of higher than average viability (Hamilton and Zuk, 1982). From the testosterone immunoredistribution hypothesis point of view, the interpretation of these results would be that high testosterone levels in territorial males provoke leukocyte redistribution which leave respiratory, digestive, or other systems less protected from infection (Braude et

al., 1999).

Intra-sexual differences in parasite infections can be attributed to ecological (exposure) or physiological (susceptibility) causes. Ecological causes of such individual differences in parasitic infections include differential exposures to pathogens through diet, microhabitat choice and breeding behaviour (Zuk and McKean, 1996). The most plausible physiological (hormonal) explanations of such differences in parasitic infection are either the indirect effects of stress on the immune system, the direct effects of sex steroids on parasite growth and development, or the indirect effects of sex steroids on parasite establishment, growth, and development within the host through effect on immune system (Zuk and McKean, 1996).

Carey (1992) and Fea et al. (1999) describe in detail the diet of the New Zealand fur seal. However, to our knowledge, no studies on the potential differences in diet between territorial and non-territorial male vertebrates are documented. This study assumed that territorial and non-territorial males had similar prey range. Food-chain transmitted parasites are positively related to food consumption. Dominance status and body weight are often related and are indirectly a function of food consumption (Krebs and Davis, 1983). Consequently, somatic growth simultaneously increases an individual's probability of reaching high rank status and the potential for exposure to the food-chain transmitted parasites. New Zealand territorial males are larger and thus may have been feeding more intensively over the months preceding the breeding season, which might have exposed them to more infective stages of all the parasite species found in the faecal samples because they are all acquired via food. In addition, NZ fur seal males fast during breeding season, which may cause nutritional and physiological stresses placing an additional pressure on the immune functions and ultimately on survival in territorial males.

The second assumption of this study is that parasitic species diversity is independent of age. While Simokova et al. (2008) show positive correlation between ectoparasite species richness and longevity among cyprinid fish species, studies in odd-toed hoofed mammals (Ezenwa et al., 2006) show a negative correlation between parasitic species diversity and host longevity, while inconsistent patterns are observed among seabird species (Hughes and Page, 2007).

The Ohau Point breeding colony is currently experiencing exponential growth with an

increase of 32% in pup production per annum (Boren et al., 2006). This rapid growth increases the number of seals in the colony and in turn increases the operational sex ratio (i.e. ratio of the number of females to number of males in the breeding colony) and the degree of polygyny as the boundary regions of territorial males at Ohau point and other sites are topographically demarcated and change little (Boren, 2005; pers. obs.). One expected outcome of the increase in the degree of polygyny is an increase in the reproductive success of a defined number of individuals that are able to defend resources (i.e. a territory) required by breeding females (Wingfield et al., 1990). Thus, the degree of sexual selection acting through male-male competition and female choice is expected to also increase (Clutton-Brock et al., 1997b). Finally, male parasitism and male-biased mortality are expected to increase with the degree of sexual selection in polygynous species (Zuk and McKean, 1996, Moore and Wilson, 2002). The direct consequence of these interdependent downstream effects is an increase in the rate of territorial male turnover (Figure 7). The trade-off between reproductive effort and the functioning of the immune system opens a new dimension to the role of parasites in regulating genetic diversity among polygynous species. Future work will consist in using mathematical models to test the role of parasites in maintaining genetic diversity in polygynous systems. Table 2 summarizes the potential impact on the health of New Zealand fur seal males facing high levels of parasite burdens. This study showed intra-sexual differences in sexual behaviour, testosterone levels, and parasitism (Figure 3, 4 and 6,, Table 1). Testosterone levels, reproductive efforts and parasite infections were higher in territorial males conferring these males with an increase in reproductive success, but may result in impaired survival.

Parasites impose an important natural selection pressures on hosts and hence have an important role in sexual selection of their hosts. Understanding microevolutionary processes is fundamental in light of the ongoing anthropogenic alteration of the environment through climatic variations and habitat reduction which ultimately affect opportunity for sexual selection and shape the life history trade-offs.

5.5 ACKNOWLEDGMENTS

Thank you to Dr. Laura Boren, Sacha Dowell, Christopher Muller, Rachel Lord, Christina Hoefsmit for their assistance in the field. Thank you to Françoise Maréchal for her precious

advice and Professor Bertrand Losson (Université de Liège, Belgium) for enabling us to carry out the scatology analyses at the Faculty of Veterinary Medicine, Department of Infectious and Parasitic Diseases, Université de Liège, Belgium. This study has been funded by the University of Canterbury (UC) Master's and Doctoral Scholarship and the New Zealand International Doctoral Research Scholarship.

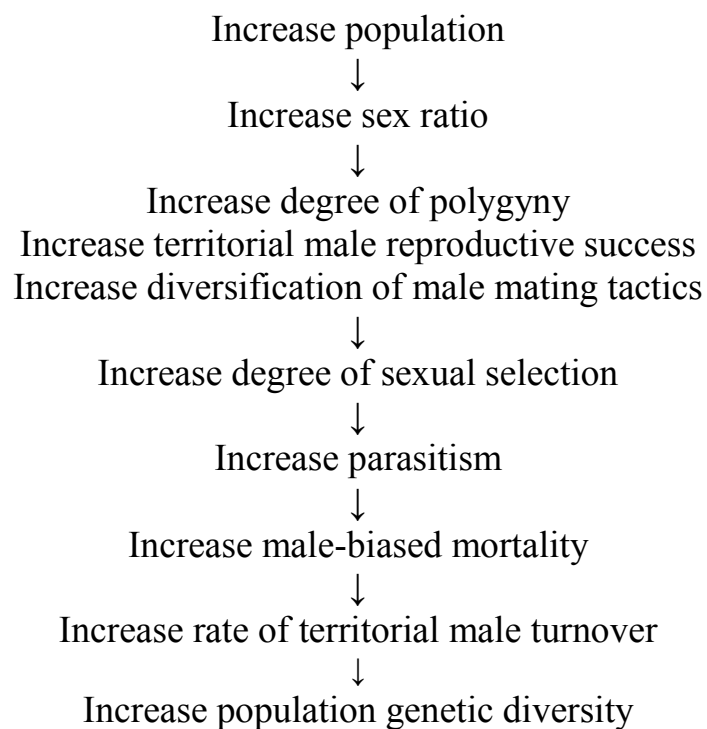


Figure 7: Downstream effects of an increase in population size in polygynous species.

Chapter Six

Summary and conclusions

6 Summary and conclusions

My thesis project primarily focused on the description of the male mating tactics and the estimation of their reproductive success in a dimorphic polygynous mammal, the New Zealand fur seal (Chapter 4). The second part of my thesis project aimed to provide insights into the costs of reproduction through estimating health parameters such as testosterone levels and helminth parasite burdens (Chapter 5). The study of reproductive success brought me to scrutinize the subtle functionalities of parentage allocation programs (i.e. CERVUS and PASOS) and the intrinsic biases (extra-matching alleles and shared alleles) in likelihood assignment approaches and to propose solutions to reduce incorrect assignment decisions (Chapter 2 and 3).

Several factors affect paternity assignment by parental allocation programs: the proportion of candidate males sampled, the level of genotypic error, the number of sampled and unsampled mothers, the level of relatedness among the samples, the level of statistical confidence applied, the intrinsic biases inherent in the likelihood methods, and the power of the assay. Categorical parentage assignment is still widely used in molecular ecological investigation with CERVUS already cited 1211 times on the Web of Science (12 December 2008). Despite the wide usage of CERVUS and its allies none of the parentage studies in the literature have investigated the effects of the user-defined parameters on the parental allocations and the combination of the results of several parentage programmes to raise correct parental allocation probability. CERVUS is very sensitive to the estimate of the total number of candidate males. The critical Δ value and the success rate were heavily dependent on the degree of overlap of the pair of distributions of Δ scores which in turn was heavily dependent on the number of candidate males. The level of confidence decreased when the rate of scoring error increased. The presence of relatives in the sampled population can be problematic. False father assignments increased when relatives in the study population increased. Activating the relatedness parameter in CERVUS decreased the percentage of false father assignments, but also decreased the percentage of true father assignments (Chapter 2). Combining two parental allocation programmes increased the reliability of parentage estimation but under-estimated the true proportion of offspring allocation.

The offspring-parent matching alleles and rare allele biases in CERVUS and PASOS constitute bias only when the number of shared rare alleles and extra matching alleles are over-weighted to the point it consistently falsely assigns parentage. These biases affected the parental assignment towards heterozygote males with rare alleles shared with the offspring (Chapter 3). Consequently, where two males could both be biological fathers of a given offspring, parentage assignment will more often go to the male with the rarer alleles (most often in heterozygous loci). Thus, the commonly used parentage assignment methods may systematically bias the results of parentage analyses towards supporting the notion that females prefer more genetically unusual, most often heterozygous, males. Such a bias may sway investigators towards incorrectly supporting the concept that females choose genetically more unusual males for heterozygosity fitness benefits that underpin the good genes hypothesis, when in fact no such relationship may exist.

Incorrect settings in the user-defined parameters and incorrect assignment decisions can affect the estimate of quantitative genetic parameters such as the variance in reproductive success among different groups. We proposed the following solutions to the above problems:

User-defined parameters (Chapter 2)

If an accurate estimate of the number of candidate parents cannot be obtained, we advocate using a computer programme to estimate the number of candidate parents such as PASOS, Patri, and MasterBayes which uses a Bayesian approach. Alternatively, Araki and Blouin's (2005) mathematical methods can estimate the number of missing parents. The microsatellite genotyping error rate can be calculated by re-genotyping a portion of the total number of samples or by measuring the mother-offspring pairs mismatches when mothers are known, prior to any analyses of parentage. If relatives are present in the sample, combining assignment of paternity and maternity lowers the bias that a related parental population induces, so that concerns arise only when the parental population is related to the offspring as full siblings.

Biases in parentage assignment and assignment decision (Chapter 3)

If multiple males are genetically compatible with an offspring tested, we first advocate accounting for demographic, sexual and behavioural information as well as logic and

heuristic methods treated as prior information in a Bayesian analysis to limit the number of potential offspring-parent relationships considered. We also advocate assignment decision on a case-by-case basis, taking into account the number of male-offspring pair and/or male-mother-offspring triad mismatches (i.e. excluding the potential father having two or more mismatches with the offspring). Finally, the number of offspring-parent matching alleles and rare alleles should be taken into account (i.e. excluding the potential father having 3 or more extra-matching alleles and/or shared rare alleles).

We implemented the insights gained from Chapter 2 and 3 in a study of male reproductive success and mating tactics in *Arctocephalus forsteri* (Chapter 4), predicting that 1) competition for females is likely to cause a diversification of male mating tactics; and 2) that alternative tactics can yield reproductive success. Our results indicated three male behavioural profiles; one corresponded to the well-recognised dominant strategy of large territorial males and two illustrated a continuum of alternative tactics employed by non-territorial subordinate males. The reproductive success of the territorial (class 1), non-territorial visitor (class 2), and non-territorial resident were 4.3, 0.5, and 1 pup(s) respectively. Our study highlights that holding a territory is not a necessary condition for reproductive success in a population of otariids.

We examined the intra-sexual differences in the costs of reproduction in male *A. forsteri* related to their reproductive success using a multidisciplinary approach that melded paternity analysis, behavioural observation, endocrinology and parasitology (Chapter 5). We showed that territorial dominant *A. forsteri* males appear to endure higher reproductive costs due to the direct and indirect effects of high testosterone levels and parasite burdens. Our study highlights that holding a territory confers a higher reproductive success, but induces higher reproductive costs that may impair survival.

6.1 PERSPECTIVES

The study was located at Ohau Point, 26 km north from Kaikoura township on the east coast. The Kaikoura coast is unusual in that it hosts an exceptionally diverse and large marine mammal fauna. The head of the Kaikoura Canyon provides a predictable and proximate food source, located around 500m off the coast, and the canyon reaches depths exceeding 1000m

around 1km off the coast. *Arctocephalus forsteri* is experiencing exponential population growth and is obviously well below its carrying capacity (Boren et al., 2006). This provides us an exciting perspective for studying the evolution of life history trade-offs through a long-term study of a population in expansion.

In contrast the New Zealand sea lion *Phocarctos hookeri* is currently in decline or is experiencing neutral population growth. Endangered species generally have small population sizes and reduced genetic variation (and consequent increased homozygosity). Low genetic variation in endangered species might play an important role in the host susceptibility to infectious agents. *Phocarctos hookeri* has experienced three recent epizootics (i.e. 1997/1998, 2001/2002, and 2002/2003) primarily cause by bacterial infections (Wilkinson et al., 2006). *Phocarctos hookeri* pups have been shown to be heavily affected by hookworm infection accounting for 16.4% of juvenile mortality per annum and in nonepidemic years (Castinel et al., 2007). In contrast, *A. forsteri* pups show no difference in survivorship between anthelmintic treated pups and untreated pups and it appears that hookworm has little effect in this species (Boren, 2005). Genetic composition and diversity between these species might be an important factor in host resistance to infectious agents. Thus, a better knowledge of growth and reproduction in a successful population such as *A. forsteri* may provide insight into populations currently in decline such as *P. hookeri* and contribute to the long term survival of this and related species by providing information to guide management decisions.

The genetic data accumulated from *A. forsteri* during this study and genotypes (from 12 microsatellites) from 2000 *P. hookeri* individuals (from pre- and post-epidemics; 1997-2007), which I have collected as an aside project, can be combined with concurrent studies at the same breeding sites to address correlation between the level of genetic diversity, pathogen resistance, and pup growth rate in *A. forsteri* and *P. hookeri*. The comparison between these species will give the opportunity to identify microsatellite markers associated with parasite resistance (*A. forsteri*) or parasite susceptibility (*P. hookeri*) and growth (data available from concurrent studies at the same breeding colonies).

REFERENCES

- ALCORN, D. J. (1984) The Hawaiian monk seal on Laysan Island, 1982. *NOAA Tech. Mem. NMFS-SNFC NMFSS WFC-42, National Marine Fisheries Service, Honolulu, HI.*
- ALDENDERFER, M. S. & BLASHFIELD, R. K. (1984) Cluster Analysis. *Sage Publication Inc.*
- ALTMANN, J. (1974) Observational study of behavior: sampling methods. *Behaviour*, 49, 227-67.
- AMBS, S. M., BONESS, D. J., BOWEN, W. D., PERRY, E. A. & FLEISCHER, R. C. (1999) Proximate factors associated with high levels of extraconsort fertilization in polygynous grey seals. *Animal Behaviour*, 58, 527-535.
- AMOS, W., TWISS, S., POMEROY, P. P. & ANDERSON, S. S. (1993) Male mating success and paternity in the grey seal, *Halichoerus grypus*: a study using DNA fingerprinting. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 252, 199-207.
- ANDERSON, S. S. & FEDAK, M. A. (1985) Grey seal males: energetic and behavioural links between size and sexual success. *Animal Behaviour*, 33, 829-838.
- ARAKI, H. & BLOUIN, M. S. (2005) Unbiased estimation of relative reproductive success of different groups: evaluation and correction of bias caused by parentage assignment errors. *Molecular Ecology*, 14, 4097-4109.
- ARNBOM, T. A., LUNN, N. J., BOYD, I. L. & BARTON, T. (1992) Aging live Antarctic fur seals and southern elephant seals. *Marine Mammal Science*, 8, 57-43.
- ARNOULD, J. P. Y. (2002) Southern fur seals. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds), 1146-1151.
- ARNOULD, J. P. Y. & DUCK, C. D. (1997) The cost and benefits of territorial tenure, and factors affecting mating success in male Antarctic fur seals. *Journal of Zoology*, 241, 649-664.
- ATKINSON, S. (1997) Reproductive biology of seals. *Rev Reprod*, 2, 175-194.
- ATKINSON, S. (2002) Male reproductive systems. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds), 700-704.
- ATKINSON, S. & GILMARTIN, W. G. (1992) Seasonal testosterone pattern in Hawaiian monk seals (*Monachus schauinslandi*). *J Reprod Fertil*, 96, 35-9.
- BAKER, W. H. (1960) Element of photogrammetry. *The Ronald Press Company, New York.*
- BARTHOLOMEW, G. A. (1952) Reproductive and social behaviour in the northern elephant seal. *University of California Publication Zoology*, 47, 369-472.
- BARTHOLOMEW, G. A. (1953) Behavioral factors affecting social structure in the Alaska fur seal. *Trans 18th N American Wildlife Conference*, 481-502.
- BARTHOLOMEW, G. A. (1970) A model for the evolution of pinniped polygyny. *Evolution*, 24, 546-559.
- BENGSTON, J. L. & LAWS, R. M. (1985) Trends in crabeater seal age at sexual maturity: an insight into Antarctic marine interactions. In *"Antarctic Nutrient Cycles and Food Webs"*. Siegfried W.R., Condy P.R., Laws, R.M. (eds). Springer, Berlin, 667-675.
- BERNATCHEZ, L. & DUCHESNE, P. (2000) Individual-based genotype analysis in studies of parentage and population assignment: how many loci, how many alleles? *Canadian Journal of Fisheries and Aquatic Sciences*, 57, 1-12.
- BERTA, A., SUMICH, J. & KOVACS, K. M. (2006) Reproductive structures, strategies, and patterns. In *"Marine mammals: evolutionary biology"*. Berta, A., Sumich, J., Kovacs, K.M. (eds). 2nd Edition, Academic Press, 363-415.

- BONESS, D. J. (1991a) Determinants of mating systems in the Otariidae (Pinnipedia). In *"Behaviour of Pinnipeds"*. Renouf, D. (ed). Chapman and Hall, London, 1-44.
- BONESS, D. J. (1991b) Determinants of mating systems in the Otariidae (Pinnipedia). In *"The Behaviour of Pinnipeds"*. Renouf, D. (ed). Chapman and Hall, New York, 1-44.
- BONESS, D. J., ANDERSON, S. S. & COX, C. R. (1982) Functions of female aggression during the pupping and mating season of gray seals, *Halichoerus grypus* (Fabricius). *Canadian Journal of Zoology*, 60, 2270-2278.
- BONESS, D. J. & BOWEN, W. D. (1996) The evolution of maternal care in pinnipeds. *BioScience*, 46, 645-654.
- BONESS, D. J., BOWEN, W. D., BUHLEIER, B. M. & MARSHALL, G. J. (2006) Mating tactics and mating system of an aquatic-mating pinniped: the harbor seal, *Phoca vitulina*. *Behavioral Ecology and Sociobiology*, 61, 119-130.
- BONESS, D. J. & JAMES (1979) Reproductive behaviour of the grey seal (*Halichoerus grypus*) on Sable Island, Nova Scotia. *Journal of Zoology, London*, 188, 477-500.
- BOREN, L. J. (2005) New Zealand fur seals in the Kaikoura region: colony dynamics, maternal investment and health. *PhD thesis, University of Canterbury, Christchurch, New Zealand*.
- BOREN, L. J., MULLER, C. G. & GEMMELL, N. J. (2006) Colony growth and pup condition of the New Zealand fur seal (*Arctocephalus forsteri*) on the Kaikoura coastline compared with other east coast colonies. *Wildlife Research*, 33, 497-505.
- BOWEN, W. D. (1991) Behavioural ecology of pinniped neonates. In *"Behaviour of Pinnipeds"*. Renouf, D. (ed). Chapman and Hall and Cambridge University Press, Cambridge, 66-127.
- BOWEN, W. D., BECK, C. A. & AUSTIN, D. A. (2002) Pinniped ecology. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds), 911-921.
- BOWEN, W. D., ELLIS, S. L., IVERSON, S. J. & BONESS, D. J. (2001) Maternal effects on offspring growth rate and weaning mass in harbour seals. *Canadian Journal of Zoology*, 79, 1088-1101.
- BOYD, I. L. (1998) Time and Energy Constraints in Pinniped Lactation. *The American Naturalist*, 152, 717-728.
- BOYD, I. L. (2002) Pinniped life history. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds), 929-934.
- BRADSHAW, C. J. (1999) Population dynamics and colonisation modelling of New Zealand fur seals. *PhD Thesis, Department of Zoology, University of Otago, Dunedin, New Zealand*.
- BRADSHAW, C. J. A., DAVIS, L. S., LALAS, C. & HARCOURT, R. G. (2000) Geographic and temporal variation in the condition of pups of the New Zealand fur seal (*Arctocephalus forsteri*): evidence for density dependence and differences in the marine environment. *Journal of Zoology*, 252, 41-51.
- BRADSHAW, C. J. A., THOMPSON, C. M., DAVIS, L. S. & LALAS, C. (1999) Pup density related to terrestrial habitat use by New Zealand fur seals. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 77, 1579-1586.
- BROWN, J. L. (1997) A theory of mate choice based on heterozygosity. *Behavioral Ecology*, 8, 60-65.
- BURCZYK, J., ADAMS, W. T., BIRKES, D. S. & CHYBICKI, I. J. (2006) Using genetic markers to directly estimate gene flow and reproductive success parameters in plants on the basis of naturally regenerated seedlings. *Genetics*, 173, 363-372.
- CAMPAGNA, C., LE BOEUF, B. J. & CAPPOZZO, H. L. (1988) Group Raids: a Mating

- Strategy of Male Southern Sea Lions. *Behaviour*, 105, 224-248.
- CAREY, P. W. (1989) Behavioural thermoregulation and polygyny in the New Zealand fur seal. *PhD thesis, University of Canterbury, Christchurch, New Zealand*.
- CAREY, P. W. (1991) Resource-defense polygyny and male territory quality in the New Zealand fur seal. *Ethology*, 88, 63-79.
- CASTINEL, A., DUIGNAN, P., POMROY, W., LYONS, E., NADLER, S., DAILEY, M., WILKINSON, I. & CHILVERS, B. (2006) First report and characterization of adult *Uncinaria* spp. in New Zealand Sea Lion (*Phocarctos hookeri*) pups from the Auckland Islands, New Zealand. *Parasitology Research*, 98, 304-309.
- CASTINEL, A., DUIGNAN, P. J., POMROY, W. E., PEZ-VILLALOBOS, N., GIBBS, N. J., CHILVERS, B. L. & WILKINSON, I. S. (2007) Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *Journal of Wildlife Diseases*, 43, 461-474.
- CAUDRON, A. K. (1997) Pinnipeds social systems: a review. *Mammalia*, 61, 153-160.
- CAUDRON, A. K., JOIRIS, C. R. & RUWET, J. C. (2001) Comparative activity budget among grey seal (*Halichoerus grypus*) breeding colonies - the importance of marginal populations. *Mammalia*, 65, 373-382.
- CAUDRON, A. K., NEGRO, S. S., FOWLER, M., ROBERTSON, B. C., PONCIN, P. & GEMMELL, N. J. (In prepa.) Alternative mating strategies in the New Zealand fur seal *Arctocephalus forsteri*: When non-territorial males are successful too.
- CAUDRON, A. K., NEGRO, S. S., MULLER, C., BOREN, L. & GEMMELL, N. J. (2007) Hair sampling and genotyping from follicles : a minimally-invasive alternative for genetics studies in small, mobile pinnipeds and other mammals. *Marine Mammal Science*, 23, 184-192.
- CERCHIO, S., JACOBSEN, J. K., CHOLEWIAK, D. M., FALCONE, E. A. & MERRIWETHER, A. D. (2005) Paternity in Humpback whales, *Megaptera novaeangliae*: assessing polygyny and skew in male reproductive success. *Animal Behaviour*, 70, 267-277.
- CHAKRABORTY, R., SHAW, M. & SCHULL, W. J. (1974) Exclusion of paternity: the current of the art. *American Journal of Human Genetics*, 26, 477-488.
- CLARK, M. M., DESOUSA, D., VONK, J. & GALEF, B. G. (1997) Parenting and potency: alternative routes to reproductive success in male Mongolian gerbils. *Animal Behaviour*, 54, 635-642.
- CLUTTON-BROCK, T. H. (1989) Mammalian mating systems. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 236, 339-372.
- CLUTTON-BROCK, T. H. & HARVEY, P. H. (1978) Mammals, resources and reproductive strategies. *Nature, London*, 273, 191-195.
- COHAS, A., YOCCOZ, N. & ALLAINÉ, D. (2007) Extra-pair paternity in alpine marmots, *Marmota marmota* : genetic quality and genetic diversity effects. *Behavioral Ecology and Sociobiology*, 61, 1081-1092.
- COHAS, A., YOCCOZ, N., DA SILVA, A., GOOSSENS, B. & ALLAINÉ, D. (2006) Extra-pair paternity in the monogamous alpine marmot (*Marmota marmota*): the roles of social setting and female mate choice. *Behavioral Ecology and Sociobiology*, 59, 597-605.
- COLTMAN, D. W., BANCROFT, D. R., ROBERTSON, A., SMITH, J. A., CLUTTON-BROCK, T. H. & PEMBERTON, J. M. (1999a) Male reproductive success in a promiscuous mammal: behavioural estimates compared with genetic paternity. *Molecular Ecology*, 8, 1199-1209.
- COLTMAN, D. W., BOWEN, W. D. & WRIGHT, J. M. (1998) Male mating success in an

- aquatically mating pinniped, the harbour seal (*Phoca vitulina*), assessed by microsatellite DNA markers. *Molecular Ecology*, 7, 627-38.
- COLTMAN, D. W., BOWEN, W. D. & WRIGHT, J. M. (1999b) A multivariate analysis of phenotype and paternity in male harbor seals, *Phoca vitulina*, at Sable Island, Nova Scotia. *Behav. Ecol.*, 10, 169-177.
- CONRAD, K. F., CLARKE, M. F., ROBERTSON, R. J. & BOAG, P. T. (1998) Paternity and the relatedness of helpers in the cooperatively breeding bell miner. *Condor*, 100, 343-349.
- CRAIG, M. P. & RAGEN, T. J. (1999) Body size, survival, and decline of juvenile Hawaiian monk seals, *Monachus schauinslandi*. *Marine Mammal Science*, 15, 786-809.
- CRAWLEY, M. C. & WILSON, G. J. (1976) The natural history of the New Zealand fur seal (*Arctocephalus forsteri*). *Tuatara*, 22, 1-29.
- DAILEY, M. D. (2001) Parasitic diseases. In *"CRC Handbook of Marine Mammal Medicine". Second Edition*. Dierauf L.A., Gulland, F.M.D. (eds). CRC Press, Boca Raton, FL, 357-379.
- DARWIN, C. R. (1871) The descent of man and selection in relation to sex. *Appleton, New York*.
- DAVIES, N. B. (1985) Cooperation and conflict among dunnocks, *Prunella modularis* in a variable mating system. *Animal Behaviour*, 33, 628-648.
- DAWKINS, R. (1980) Good strategy or evolutionary stable strategy? In *"Sociobiology: beyond nature/nurture?"* Barlow, G. W. (ed) Silverberg, Westview, Boulder, CO, 331-367.
- DEVLIN, B., ROEDER, K. & ELLSTRAND, N. C. (1988) Fractional Paternity Assignment - Theoretical Development and Comparison to Other Methods. *Theoretical and Applied Genetics*, 76, 369-380.
- DOWELL, S. A., BOREN, L., NEGRO, S. S., MULLER, C. G., CAUDRON, A. K. & GEMMELL, N. J. (2008) Rearing two New Zealand fur seal (*Arctocephalus forsteri*) pups to weaning. *Australian Journal of Zoology*, 56, 33-39.
- DUCHESNE, P. & BERNATCHEZ, L. (2007) Individual-based genotype methods in aquaculture. In *"Aquaculture genome technologies"*. Zhanjiang Liu (Ed.), Auburn University., 87-108.
- DUCHESNE, P., CASTRIC, T. & BERNATCHEZ, L. (2005) PASOS (parental allocation of singles in open systems): a computer program for individual parental allocation with missing parents. *Molecular Ecology Notes*, 5, 701.
- EMLEN, S. T. & ORING, L. W. (1977) Ecology, sexual selection, and the evolution of mating systems. *Science*, 197, 215-223.
- EVETT, I. W. & WEIR, B. S. (1998) Interpreting DNA evidence. *Sinauer Associates, Sunderland, MA*, 115-116.
- EXCOFFIER, L. & HECKEL, G. (2006) Computer programs for population genetics data analysis: a survival guide. *Nature Reviews. Genetics*, 7, 745-758.
- FAUQUIER, D., GULLAND, F., HAULENA, M., DAILEY, M., RIETCHECK, R. L. & LIPSCOMB, T. P. (2004) Meningoencephalitis in Two Stranded California Sea Lions (*Zalophus californianus*) Caused by Aberrant Trematode Migration. *Journal of Wildlife Diseases*, 40, 816-819.
- FLETCHER, D., GULLAND, F. M. D., HAULENA, M., LOWENSTINE, L. J. & DAILEY, M. (1998) Nematode-associated gastrointestinal perforations in stranded California sea lions (*Zalophus californianus*). In *"International Association for Aquatic Animal Medicine". 29th Annual Conference Proceedings, San Diego, CA*, 59.
- FLYNN, J. J., FINARELLI, J. A., ZEHR, S., HSU, J. & NEDBAL, M. A. (2005) Molecular

- phylogeny of the Carnivora (Mammalia): assessing the impact of increased sampling on resolving enigmatic relationships. *Systematic Biology*, 54, 317-337.
- FOERSTER, K., DELHEY, K., JOHNSEN, A., LIFJELD, J. T. & KEMPENAERS, B. (2003) Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature*, 425, 714-717.
- FOLSTAD, I. & KARTER, A. J. (1992) Parasites, Bright Males, and the Immunocompetence Handicap. *The American Naturalist*, 139, 603.
- FOREYT, W. J. (1997) Veterinary Parasitology Reference Manual. *College of Veterinary Medicine, Washington State University, Pullman, WA*.
- FOSSOY, F., JOHNSEN, A. & LIFJELD, J. T. (2007) Multiple genetic benefits of female promiscuity in a socially monogamous passerine. *Evolution*, 62, 145-156.
- FOWLER, M. (2003) Spatial distribution of male New Zealand fur seals, *Arctocephalus forsteri*, during the breeding season. *Honour's thesis, Southampton College, Long Island University, New York, USA*.
- GALES, N. J., HABERLEY, B. & COLLINS, P. (2000) Changes in the abundance of New Zealand fur seals, *Arctocephalus forsteri*, in Western Australia. *Wildlife Research*, 27, 165-168.
- GARNER, D. (1953) Physical characteristics of inshore surface waters between Cook Strait and Banks Peninsula, New Zealand. *New Zealand Journal of Science and Technology*, 35, 239-246.
- GELATT, T. S. (2001) Male reproductive success, relatedness, and the mating system of Weddell seals in McMurdo Sound, Antarctica. *PhD Thesis, University of Minnesota, St. Paul, MN*.
- GEMMELL, N. J., BURG, T. M., BOYD, I. L. & AMOS, W. (2001) Low reproductive success in territorial male Antarctic fur seals (*Arctocephalus gazella*) suggests the existence of alternative mating strategies. *Molecular Ecology*, 10, 451-60.
- GEMMELL, N. J. & MAJLUF, P. (1997) Projectile biopsy sampling of fur seals. *Marine Mammal Science*, 13, 512-516.
- GENTRY, R. L. (1975) Comparative social behaviour of the eared seals. *Rapp. P.-v. Réun. Cons. Int. Explor. Mer*, 169, 188-194.
- GENTRY, R. L. & KOOYMAN, G. L. (1982) Fur seals: maternal strategies on land and at sea. *Princeton University Press*.
- GERBER, S., MARIETTE, S., STREIFF, R., BODENES, C. & KREMER, A. (2000) Comparison of microsatellites and amplified fragment length polymorphisms markers for parentage analysis. *Molecular Ecology*, 9, 1037-1048.
- GOLDSWORTHY, S. D., BONESS, D. J. & FLEISCHER, R. C. (1999) Mate choice among sympatric fur seals: female preference for conspecific males. *Behavioral Ecology and Sociobiology*, 45, 253-267.
- GOLDSWORTHY, S. D. & SHAUGHNESSY, P. D. (1994) Breeding biology and haul-out pattern of the New Zealand fur seal (*Arctocephalus forsteri*) at Cape Gantheaume, South Australia. *Wildlife Research*, 21, 365-376.
- GROSS, M. R. (1996) Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology & Evolution*, 11, 92-98.
- GROSSMAN, C. J. (1985) Interactions between the gonadal steroids and the immune system. *Science*, 227, 257-261.
- GULLAND, F. M. D., BECKMEN, K., BUREK, K., LOWENSTINE, L., WERNER, L., SPRAKER, T., DAILEY, M. & HARRIS, E. (1997) Nematode (*Otostrongylus circumlitus*) infection of northern elephant seals (*Mirounga angustirostris*) stranded along the central California coast. *Marine Mammal Science*, 13, 446-458.

- HADFIELD, J. D., RICHARDSON, D. S. & BURKE, T. (2006) Towards unbiased parentage assignment: combining genetic, behavioural and spatial data in a Bayesian framework. *Molecular Ecology*, 15, 3715-3730.
- HALEY, M. P., DEUTSCH, C. J. & LE BOEUF, B. J. (1991) A method for estimating mass of large pinnipeds. *Marine Mammal Science*, 7, 157-164.
- HANGGI, E. B. & SCHUSTERMAN, R. J. (1994) Underwater acoustic displays and individual variation in male harbour seals, *Phoca vitulina*. *Animal Behaviour*, 48, 1275-1283.
- HARCOURT, R. (2001) Advances in New Zealand mammalogy 1990-2000: pinnipeds. *Journal of the Royal Society of New Zealand*, 31, 135-160.
- HARCOURT, R., KINGSTON, J., CAMERON, M., WAAS, J. & HINDELL, M. (2007) Paternity analysis shows experience, not age, enhances mating success in an aquatically mating pinniped, the Weddell seal (*Leptonychotes weddellii*). *Behavioral Ecology and Sociobiology*, 61, 643-652.
- HARCOURT, R. G., BRADSHAW, C. J. A., DICKSON, K. & DAVIS, L. S. (2002) Foraging ecology of a generalist predator, the female New Zealand fur seal. *Marine Ecology Progress Series*, 227, 11-24.
- HARCOURT, R. G., KINGSTON, J. J., WAAS, J. R. & HINDELL, M. A. (2008) Foraging while breeding: Alternative mating strategies by male Weddell seals? *Aquatic Conservation: Marine and Freshwater Ecosystems*, 17, S68-S78.
- HARCOURT, R. G., SCHULMAN, A. M., DAVIS, L. S. & TRILLMICH, F. (1995) Summer foraging by lactating female New Zealand fur seals (*Arctocephalus forsteri*) off Otago Peninsula, New Zealand. *Canadian Journal of Zoology*, 73, 678-690.
- HAYES, S. A., PEARSE, D. E., COSTA, D. P., HARVEY, J. T., LE BOEUF, B. J. & GARZA, J. C. (2006) Mating system and reproductive success in eastern Pacific harbour seals. *Molecular Ecology*, 15, 3023-3034.
- HEATH, C. B. (2002) California, Galapagos, and Japanese sea lions. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds). Academic Press, San Diego, 180-186.
- HECKEL, G. & VON HELVERSEN, O. (2002) Male tactics and reproductive success in the harem polygynous bat *Saccopteryx bilineata*. *Behav. Ecol.*, 13, 750-756.
- HOBY, S., SCHWARZENBERGER, F., DOHERR, M. G., ROBERT, N. & WALZER, C. (2006) Steroid hormone related male biased parasitism in chamois, *Rupicapra rupicapra*. *Veterinary Parasitology*, 138, 337-348.
- HOELZEL, A. R., LE BOEUF, B. J., REITER, J. & CAMPAGNA, C. (1999) Alpha-male paternity in elephant seals. *Behavioral Ecology and Sociobiology*, 46, 298-306.
- HOFFMAN, J. I. & AMOS, W. (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. *Molecular Ecology*, 14, 599-612.
- HOFFMAN, J. I., BOYD, I. L. & AMOS, W. (2003) Male reproductive strategy and the importance of maternal status in the antarctic fur seal *Arctocephalus gazella*. *Evolution*, 57, 1917-1930.
- HOFFMAN, J. I., BOYD, I. L. & AMOS, W. (2004) Exploring the relationship between parental relatedness and male reproductive success in the antarctic fur seal *Arctocephalus gazella*. *Evolution*, 58, 2087-2099.
- HOFFMAN, J. I., FORCADA, J., TRATHAN, P. N. & AMOS, W. (2007) Female fur seals show active choice for males that are heterozygous and unrelated. *Nature*, 445 (7130): 912-4.
- HOGG, J. T. & FORBES, S. H. (1997) Mating in bighorn sheep: frequent male reproduction

- via a high-risk “unconventional” tactic. *Behavioral Ecology and Sociobiology*, 41, 33-48.
- HUGHES, C. (1998) Integrating Molecular Techniques with Field Methods in Studies of Social Behavior: A Revolution Results. *Ecology*, 79, 383-399.
- IONITA, M., VARELA, M. G., LYONS, E. T., SPRAKER, T. R. & TOLLIVER, S. C. (2008) Hookworms (*Uncinaria lucasi*) and acanthocephalans (*Corynosoma* spp. and *Bolbosoma* spp.) found in dead northern fur seals (*Callorhinus ursinus*) on St. Paul Island, Alaska in 2007. *Parasitology Research*, 103, 1025-1029.
- JAMIESON, A. & TAYLOR, S. C. S. (1997) Comparisons of three probability formulae for parentage exclusion. *Animal Genetics*, 28, 397-400.
- JOHNSON, S. C. (1967) Hierarchical clustering schemes. *Psychometrika*, 32, 241-254.
- JONES, A. & ARDREN, W. (2003) Methods of parentage analysis in natural populations. *Molecular Ecology*, 12, 2511-2523.
- KALINOWSKI, S. T., RAPER, M. L. & MARSHALL, T. C. (2007) Revising how the computer program CERVUS accomodates genotyping error increases success in paternity assignment. *Molecular Ecology*, 16, 1099-1106.
- KEMPENAERS, B. (2007) Mate choice and genetic quality: a review of the heterozygosity theory. *Advances in the Study of Behavior*, 37, 189-278.
- KIYOTA, M. (2005) Site fidelity, territory acquisition and mating success in male northern fur seals (*Callorhinus ursinus*). *Mammal Study*, 30, 19-27.
- KOVACS, K. M. & LAVIGNE, D. (1992) Maternal investment in otariid seals and walruses. *Canadian Journal of Zoology*, 70, 1953-1964.
- KREBS, C. J. (2001) Ecology: the experimental analysis of distribution and abundance. 5th edition. San Francisco: Benjamin Cummings.
- KUNC, H. P. & WOLF, J. B. W. (2008) Seasonal changes of vocal rates and their relation to territorial status in male Galápagos sea lions (*Zalophus wolfebaeki*). *Ethology*, 114, 381-388.
- LALAS, C. & BRADSHAW, C. J. (2001) Folklore and chimerical numbers: review of a millennium of interaction between fur seals and humans in the New Zealand region. *New Zealand Journal of Marine and Freshwater Research*, 35, 477-497.
- LANCASTER, M. L., GEMMELL, N. J., NEGRO, S., GOLDSWORTHY, S. & SUNNUCKS, P. (2006) Ménage à trois on Macquarie Island: hybridization among three species of fur seal (*Arctocephalus* spp.) following historical population extinction. *Molecular Ecology*, 15, 3681-3692.
- LANCASTER, M. L., GOLDSWORTHY, S. D. & SUNNUCKS, P. (2007) Multiple mating strategies explain unexpected genetic mixing of New Zealand fur seals with two congenics in a recently recolonized population. *Molecular Ecology*, 16, 5267-5276.
- LAUCKNER, G. (1985) Diseases of mammalia: Pinnipeda. In "Diseases of Marine Animals". Vol. IV, Pt. 2, Kinne, O. (ed). Biologische Anstalt Helgoland, Hamburg, chap. 5, 683-793.
- LE BOEUF, B. J. (1974) Male-male Competition and Reproductive Success in Elephant Seals. *Amer. Zool.*, 14, 163-176.
- LE BOEUF, B. J. (1991) Pinniped mating systems on land, ice, and in water: emphasis on the Phocidae. In "The Behaviour of Pinnipeds". Renouf, D. (ed). Chapman and Hall, London, 45-65.
- LE BOEUF, B. J. & LAWS, R. M. (1994) Elephant seals: an introduction to the genus. In "Elephant Seals". Le Boeuf, B.J. and Laws, R.M. (eds). University of California Press, Berkeley, 1-26.
- LE BOEUF, B. J. & MESNICK, S. (1991) Sexual Behavior of Male Northern Elephant

- Seals: I. Lethal Injuries To Adult Females. *Behaviour*, 116, 143-162.
- LEHNERT, K., RAGA, J. A. & SIEBERT, U. (2007) Parasites in harbour seals (*Phoca vitulina*) from the German wadden sea between two phocine distemper virus epidemics. *Helgoland Marine Research*, 61, 239-245.
- LIDGARD, D. C., BONESS, D. J., BOWEN, W. D. & MCMILLAN, J. I. (2005) State-dependent male mating tactics in the grey seal: the importance of body size. *Behavioural Ecology*, 16, 541-549.
- LIDGARD, D. C., BONESS, D. J., BOWEN, W. D., MCMILLAN, J. I. & FLEISCHER, R. C. (2004) The rate of fertilization in male mating tactics of the polygynous grey seal. *Molecular Ecology*, 13, 3543-3548.
- LINKLATER, W. L., CAMERON, E. Z., MINOT, E. O. & STAFFORD, K. J. (1999) Stallion harassment and the mating system of horses. *Animal Behaviour*, 58, 295-306.
- LUNN, N. J., BOYD, I. L., BARTON, T. & CROXALL, J. P. (1993) Factors Affecting the Growth Rate and Mass at Weaning of Antarctic Fur Seals at Bird Island, South Georgia. *Journal of Mammalogy*, 74, 908-919.
- LYONS, E. T., DELONG, R. L., GULLAND, F. M., MELIN, S. R., TOLLIVER, S. C. & SPRAKER, T. R. (2000) Comparative Biology of *Uncinaria* spp. in the California Sea Lion (*Zalophus californianus*) and the Northern Fur Seal (*Callorhinus ursinus*) in California. *The Journal of Parasitology*, 86, 1348-1352.
- MAJLUF, P. (1987) Reproductive ecology of female South American fur seals at Punta San Juan. *PhD Thesis M-22, University of Cambridge, UK*.
- MAJLUF, P. & GOEBEL, M. E. (1992) The capture and handling of female South American fur seals and their pups. *Marine Mammal Science*, 8, 187-190.
- MARSHALL, T. C., SLATE, J., KRUUK, L. E. & PEMBERTON, J. M. (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, 7, 639-55.
- MASTERS, B. S., HICKS, B. G., JOHNSON, L. S. & ERB, L. A. (2003) Genotype and extra-pair paternity in the house wren: a rare-male effect? *Proceedings of the Royal Society B: Biological Sciences*, 270, 1393-1397.
- MATTLIN, R. (1987) New Zealand fur seal (*Arctocephalus forsteri*) within the New Zealand region. In "Status, Biology and Ecology of Fur Seals: NOAA Technical Report NMFS 51". Croxall, J. and Gentry, R. (eds). National Marine Fisheries Service, Cambridge, UK, 49-51.
- MATTLIN, R. H. (1978) Population biology, thermoregulation and site preference of the New Zealand fur seal, *Arctocephalus forsteri* (Lesson, 1828), on the Open Bay Islands, New Zealand. *PhD thesis, University of Canterbury, Christchurch, New Zealand*.
- MAYS, H., ALBRECHT, T., LIU, M. & HILL, G. (2007) Female choice for genetic complementarity in birds: a review. *Genetica*.
- MCCANN, T. S. (1980) Territoriality and breeding behaviour of adult male Antarctic fur seal, *Arctocephalus gazella*. *Journal of Zoology*, 192, 295-310.
- MCCONKEY, S. D. (1999) Photographic identification of the New Zealand sea lion: a new technique. *New Zealand Journal of Marine and Freshwater Research*, 33, 63-66.
- MCKENZIE, J., GOLDSWORTHY, S. D., SHAUGHNESSY, P. D. & MCINTOSH, R. (2005) Understanding the impediments to the growth of Australian sea lion populations. *South Australia Research and Development Institute (Aquatic Sciences), Adelaide, 107pp. SARDI Publication Number RD04/0171*.
- MCKENZIE, J., PAGE, P., SHAUGHNESSY, P. D. & HINDELL, M. A. (2007) Age and reproductive maturity of New Zealand fur seals (*Arctocephalus forsteri*) in southern

- Australia. *Journal of Mammalogy*, 88, 639-648.
- MCLAREN, J. A. (1967) Seals and group selection. *Ecology*, 48, 104-110.
- MCRAE, S. B. & KOVACS, K. M. (1994) Paternity exclusion by DNA fingerprinting, and mate guarding in the hooded seal *Cystophora cristata*. *Molecular Ecology*, 3, 101-107.
- MEAGHER, T. R. (1986) Analysis of paternity within a natural population of *Chamaelirium luteum*. I. Identification of most-likely male parents. *The American Naturalist*, 128, 199-215.
- MILLER, E. (1971) Social and thermoregulatory behaviour of the New Zealand fur seal, *Arctocephalus forsteri* (Lesson, 1828). *MSc thesis, University of Canterbury, Christchurch, New Zealand*.
- MILLER, E. H. (1974) Social behaviour between adult male and female New Zealand fur seals, *Arctocephalus forsteri* (Lesson) during the breeding season. *Australian Journal of Zoology*, 22, 155-173.
- MILLER, E. H. (1975) Social and evolutionary implications of territoriality in adult male New Zealand fur seal, *Arctocephalus forsteri* (Lesson, 1828), during the breeding season. In "Biology of seals" Ronald, K. and Mansfield, A.W. (eds). *Proceeding symposium Guelph, 1972*.
- MILLER, E. H. (1991) Communication in pinnipeds, with special reference to non-acoustic signalling. In "The Behaviour of Pinnipeds". Renouf, D. (ed). *Chapman and Hall, New York*, 128-235.
- MOLLER, A. P. & SAINO, N. (1994) Parasites, immunology of hosts, and host sexual selection. *J Parasitol*, 80, 850-8.
- MOORE, S. L. & WILSON, K. (2002) Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, 297, 2015-8.
- MORGAN, M. T. & CONNER, J. K. (2001) Using genetic markers to directly estimate male selection gradients. *Evolution*, 55, 272-281.
- NEFF, B. D., REPKA, J. & GROSS, M. R. (2000a) Parentage analysis with incomplete sampling of candidate parents and offspring. *Molecular Ecology*, 9, 515-528.
- NEFF, B. D., REPKA, J. & GROSS, M. R. (2000b) Statistical confidence in parentage analysis with incomplete sampling: how many loci and offspring are needed? *Molecular Ecology*, 9, 529-539.
- NEFF, B. D., REPKA, J. & GROSS, M. R. (2001) A Bayesian framework for parentage analysis: the value of genetic and other biological data. *Theoretical Population Biology*, 59, 315-331.
- NEGRO, S. S., CLOOTS, R. & GEMMELL, N. J. (2006) Cost-effective media for the rapid and high resolution of small DNA fragments using polyacrylamide-based electrophoresis. *Molecular Ecology Notes*, 6, 609-612.
- NEGRO, S. S., FREEMAN, I. & GEMMELL, N. J. (In Review) Bias in parentage assignment: do heterozygous males achieve greater reproductive success? *Heredity*.
- NIELSEN, R., MATTILA, D., CLAPHAM, P. J. & PALSBOLL, P. J. (2001) Statistical approaches to paternity analysis in natural populations and applications to the Northern Atlantic humpback whale. *Genetics*, 157, 1673-1682.
- OKA, T. & TAKENAKA, O. (2001) Wild gibbons' parentage tested by non-invasive DNA sampling and PCR-amplified polymorphic microsatellites. *Primates*, 42, 67-73.
- PAETKAU, D. & STROBECK, C. (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, 3, 489-495.
- PAYNE, R. B. (1979) Sexual selection and intersexual differences in variance of breeding success. *The American Naturalist*, 114, 447-452.

- PEAKALL, R. & SMOUSE, P. E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295.
- PIOZ, M., LOISON, A., GAUTHIER, D., GIBERT, P., JULLIEN, J.-M., ARTOIS, M. & GILOT-FROMONT, E. (2008) Diseases and reproductive success in a wild mammal: example in the alpine chamois. *Oecologia*, 155, 691-704.
- POMEROY, P. P., FEDAK, M. A., ROTHERY, P. & ANDERSON, S. (1999) Consequences of Maternal Size for Reproductive Expenditure and Pupping Success of Grey Seals at North Rona, Scotland. *The Journal of Animal Ecology*, 68, 235-253.
- QUELLER, D. C. & GOODNIGHT, K. F. (1989) Estimating relatedness using genetic markers. *Evolution*, 43, 258-275.
- RALLS, K. & MESNICK, S. L. (2002) Sexual dimorphism. In *"Encyclopedia of Marine Mammals"*. Perrin, W.F., Würsig, B., Thewissen, J.G.M. (eds). Academic Press, San Diego, 1071-1078.
- RASA, O. A. E. (1989) Helping in dwarf mongoose societies: an alternative reproductive strategy. In *"The sociobiology of sexual and reproductive strategies"* Rasa, O. A. E. and Vogel, C. (eds). Beckenham, Croom Helm, 61-73.
- RAYMOND, M. & ROUSSET, F. (1995) Genepop (Version 1.2) - population genetics software for exact tests of ecumenicism. *Journal of Heredity*, 86.
- READ, D. W. & LE BLANC, S. A. (2003) Population growth, carrying capacity, and conflict. *Current anthropology*, 44, 59-85.
- REBY, D., CARGNELUTTI, B. & HEWISON, A. J. M. (1999) Contexts and possible functions of barking in roe deer. *Animal Behaviour*, 57, 1121-1128.
- REED, C. A. (1965) Seals of the World. Judith E. King. British Museum (Natural History), London, 1964. viii + 154 pp. Illus. Paper, 11s. *Science*, 148, 826-a-827.
- REIJNDERS, P. J., BRASSEUR, S., VAN DE TORN, J., VAN DER WOLF, I., BOYD, I. L., HARWOOD, J., LAVIGNE, D. & LOWRY, L. (1993) Status survey and conservation action plan: seals, fur seals, sea lions, and walrus. *IUNC, Gland, Switzerland*.
- REITER, J. & BOEUF, B. (1991) Life history consequences of variation in age at primiparity in northern elephant seals. *Behavioral Ecology and Sociobiology*, 28, 153-160.
- RIDGWAY, S., GERACI, J. R. & MEDWAY, W. (1975) Diseases of pinnipeds. *Rapp. P. V. Reun. Cons. Int. Explor. Mer*, 169, 327-337.
- RIEDMAN, M. L. (1990) The pinnipeds: seals, sea lions and walruses. *University of California Press, Berkeley, LA and Oxford*.
- RITLAND, K. (2002) Extensions of models for the estimation of mating systems using n independent loci. *Heredity*, 88, 221-228.
- ROBERTSON, B. C. & GEMMELL, N. J. (2005) Microsatellite DNA markers for the study of population structure in the New Zealand fur seal (*Arctocephalus forsteri*). *DOC Science Internal Series no. 196. Department of Conservation, Wellington, New Zealand*.
- ROBERTSON, D., ROBERTS, P. & WILSON, J. (1978) Mesopelagic faunal transition across the Subtropical Convergence east of New Zealand. *New Zealand Journal of Marine and Freshwater Research*, 12, 295-312.
- RODRIGUEZ, D. & BASTIDA, R. (1998) Four hundred years in the history of pinniped colonies around Mar del Plata, Argentina. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 8, 721-735.
- RUBENSTEIN, D. I. & WRANGHAM, R. W. (1986) Socioecology: origins and trends. In *Ecological aspects of social evolution*. Rubenstein, D.I. and Wrangham, R.W. (eds).

- Princeton University Press, 3-20.
- SHAUGHNESSY, P. D., DENNIS, T. E. & SEAGER, P. G. (2005) Status of Australian sea lions, *Neophoca cinerea*, and New Zealand fur seals, *Arctocephalus forsteri*, on Eyre Peninsula and the far west coast of South Australia. *Wildlife Research*, 32, 85-101.
- SHAUGHNESSY, P. D. & MCKEOWN, A. (2002) Trends in abundance of New Zealand fur seals, *Arctocephalus forsteri*, at the Neptune Islands, South Australia. *Wildlife Research*, 29, 363-370.
- SHUSTER, S. M. & WADE, M. J. (2003) Mating systems and strategies. *Princeton University Press, Princeton and Oxford*.
- SLATE, J., MARSHALL, T. & PEMBERTON, J. (2000) A retrospective assessment of the accuracy of the paternity inference program CERVUS. *Molecular Ecology*, 9, 801-808.
- SLATER, P. J. B. (1981) Chaffinch song repertoires: observations, experiments and a discussion of their significance. *Zeitschrift für Tierpsychologie*, 56, 1-24.
- SOKAL, R. R. & ROHLF, F. J. (1995) **Biometry**. 3rd edn. *W.H. Freeman and Company, New York*.
- SOLTIS, J., MITSUNAGA, F., SHIMIZU, K., NOZAKI, M., YANAGIHARA, Y., DOMINGO-ROURA, X. & TAKENAKA, O. (1997) Sexual selection in Japanese macaques II: female mate choice and male-male competition. *Animal Behaviour*, 54, 737-746.
- ST. AUBIN, D. J. (2001) Endocrinology. In *"CRC Handbook of Marine Mammal Medicine"*. Second Edition. Dierauf L.A., Gulland, F.M.D. (eds). *CRC Press, Boca Raton, FL*, 165-192.
- STATSOFT (2005) STATISTICA (data analysis software system) version 7.1. www.statsoft.fr.
- STEWART, B. S. & DELONG, R. L. (1993) Seasonal dispersal and habitat use of foraging northern elephant seals. *Symposia of the Zoological Society of London*, 66, 179-194.
- STIRLING, I. (1970) Observations on the behaviour of the New Zealand fur seal (*Arctocephalus forsteri*). *Journal of Mammalogy*, 51, 766-778.
- THOMPSON, E. A. (1976) Paradox of genealogical inference. *Advances in Applied Probability*, 8, 648-650.
- TINBERGEN, N. (1953) Social behaviour in animals. In *"Methuen's monographs on biological subjects"*. Abercrombie (ed). *Muthuen, London, UK*.
- TRIVERS, R. L. (1972) Parental investment and sexual selection. In *"Sexual selection and the descent of man"* Campbell, B. G. (ed), Aldine-Atherton, Chicago, 136-179.
- TWISS, S. D., POLAND, V. F., GRAVES, J. A. & POMEROY, P. P. (2006) Finding fathers: spatio-temporal analysis of paternity assignment in grey seals (*Halichoerus grypus*). *Molecular Ecology*, 15, 1939-1953.
- TWISS, S. D., THOMAS, C., POLAND, V., GRAVES, J. A. & POMEROY, P. (2007) The impact of climatic variation on the opportunity for sexual selection. *Biology Letters*, 3, 12-15.
- VAN PARIJS, S. M. (2003) Aquatic mating in pinnipeds: a review. *Aquatic Mammals*, 29, 214-226.
- VAN PARIJS, S. M. & CLARK, C. W. (2006) Long-term mating tactics in an aquatic-mating pinniped, the bearded seal, *Erignathus barbatus*. *Animal Behaviour*, 72, 1269-1277.
- VAN PARIJS, S. M., LYDERSEN, C. & KOVACS, K. M. (2003) Vocalizations and movements suggest alternative mating tactics in male bearded seals. *Animal Behaviour*, 65, 273-283.

- WAINSTEIN, M. D. (2000) Paternity in southern elephant seals (*Mirounga leonina*). *PhD Thesis, University of California, Santa Cruz, USA*.
- WALSH, P. S., METZGER, D. A. & HIGUCHI, R. (1991) Chelex 100 as a medium for the simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, 10, 506-513.
- WECKERLY, F. W. (1998) Sexual-Size Dimorphism: Influence of Mass and Mating Systems in the Most Dimorphic Mammals. *Journal of Mammalogy*, 79, 33-52.
- WESTNEAT, D. F. & SHERMAN, P. W. (1997) Density and extra-pair fertilizations in birds: a comparative analysis. *Behavioral Ecology and Sociobiology*, 41, 205-215.
- WICKENS, P. & YORK, A. E. (1997) Comparative population dynamics of fur seals. *Marine Mammal Science*, 13, 241-292.
- WINGFIELD, J. C., HEGNER, R. E., DUFTY, A. M., JR. & BALL, G. F. (1990) The "Challenge Hypothesis": Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *The American Naturalist*, 136, 829-846.
- WISHART, D. (2006) ClustanGraphics primer. *A guide to cluster analysis, 4th Ed. Clustan Ltd, Edinburgh and St Andrews*.
- WORTHINGTON WILMER, J., ALLEN, P. J., POMEROY, P. P., TWISS, S. D. & AMOS, W. (1999) Where have all the fathers gone? An extensive microsatellite analysis of paternity in the grey seal (*Halichoerus grypus*). *Molecular Ecology*, 8, 1417-29.
- YOUNG, P. & LOWE, D. (1969) Larval nematodes from fish of the subfamily Anisakinae and gastrointestinal lesions in mammals. *Journal of Comparative Pathology*, 301-313.
- ZUK, M. (1996) Disease, Endocrine-Immune Interactions, and Sexual Selection. *Ecology*, 77, 1037-1042.
- ZUK, M. & MCKEAN, K. A. (1996) Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology*, 26, 1009-1024.









APPENDIX

TECHNICAL NOTE

Cost-effective media for the rapid and high resolution of small DNA fragments using polyacrylamide-based electrophoresis

S. S. NEGRO,* R. CLOOTS† and N. J. GEMMELL*

*Molecular Ecology Laboratory, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand,

†Structural Inorganic Chemistry Laboratory, BAT. B6 Department of Chemistry, Allée de la Chimie 3, 4000 Liège1, University of Liège, Liège, Belgium

Abstract

Current Tris-based solutions for DNA electrophoresis produce a positive feedback loop between current and temperature at high voltage, resulting in long running times for the separation of even small DNA fragments. We optimized the separation of small DNA fragments (90–300 bp) in polyacrylamide-based electrophoresis at high voltages (200volts/cm) by substituting Tris with low concentration alkali salts (e.g. 1 mM LiCl and CsCl). These media reduced the heat produced during electrophoresis, enhanced the DNA fragment resolution, and allowed gels to be run at higher voltages, reducing gel running times by 25%. In addition, the elimination of Tris and EDTA from the buffer reduced material costs approximately 10-fold.

Keywords: Alkali salt, gel-electrophoresis, microsatellite

Received 13 September 2005; revision received 14 November 2005; accepted 13 January 2006

Electrophoretic separation of DNA by size is a basic, but fundamental, component of modern molecular sciences, such as molecular ecology (Sanger & Coulson 1975; Sanger *et al.* 1992). Tiselius (1937) first had the idea of separating charged particles in solution using electric fields (e.g. zone boundary electrophoresis), but convection, which led to transport processes faster than electrophoresis itself, meant that this approach was not widely employed. Performing the electrophoretic separation in a gel (Grabar & Williams 1953) solved the convection problem, and Aaij & Borst (1972) further improved the method by using agarose gel instead of agar gel for separation of DNA molecules.

Modifications to the basic protocol have been few and far between over the past three decades. Tris-acetic acid-disodium EDTA (TAE) and Tris-boric acid-disodium EDTA (TBE) remain the of conductive media choice for standard DNA electrophoresis. However, Tris- and acetate-based electrophoresis present several disadvantages, of which the most significant is the existence of a positive feedback

loop related to heat and current (Brody & Kern 2004b, c). TBE and TAE buffers have high ionic strengths that lead to excessive heat generation, limiting the voltage that can be applied to the electrophoretic systems and consequently increasing DNA migration time (Brody & Kern 2004c). In addition, the high costs of Tris and the precipitation of stock solutions are other disadvantages associated with Tris- and acetate-based solutions.

Recently, Brody & Kern (2004a, b) simplified electrophoretic buffers by substituting TBE and TAE buffers for a more efficient and inexpensive conductive media in an agarose gel system. By substituting various cations and anions for Tris in the conductive media they successfully mitigated the positive feedback loop in agarose gel systems. We therefore explored the ability of simple, non-Tris-based conductive media containing different concentrations of alkali salts, to resolve small DNA fragments (90–300 bp), such as microsatellites, in a polyacrylamide-based electrophoresis system.

DNA fragments (90–300 bp) were size-fractionated on 6% denaturing polyacrylamide gels. Different concentrations of salt solutions (1, 5, and 10 mM lithium (Li) chloride acid;

Correspondence: S. S. Negro, Fax: +6433642590;

E-mail: sne23@student.centerburg.ac.nz

1 and 5 mM caesium (Cs) chloride acid) added to a sodium borate solution (SB; 10 mM sodium hydroxide, pH 8.5 adjusted with boric acid; 10 mM Na) were tested as conductive media. We omitted EDTA since it is superfluous because most DNA samples are readily soluble and the ultrapure enzymes commonly used today do not carry a detrimental enzymatic activity under electrophoretic conditions. The total volume of medium in the reservoirs of the gel rig was approximately 1100 mL and all electrophoresis runs used α - 32 P-labelled DNA fragments generated by a sequencing reaction using a forward M13 primer and pBSMB plasmid. Gels were 1 mm thick and 40 cm long. The DNA fragments were denatured at 94 °C for 3 min, placed on ice, and an equal amount of DNA (c. 600 ng) was loaded onto 6% denaturing polyacrylamide gels for each conductive medium tested. Power was set at 100 W, and voltages (120–200 V/cm) and current flow (milliamps) were recorded at 10 min intervals throughout the DNA electrophoresis separation. The temperature (°C) of the conductive media was also recorded every 10 min to observe the rate of heat generation during DNA separation for each conductive medium tested. The fragments were run for 1 h 50 min, but we found that a 1 h 40 min run was sufficient for separation of small DNA fragments (less than 150 bp). Kodak Biomax MR autoradiographic film (Radiographic Supplies) was exposed to the separated DNA fragments for 3 days and the resulting fragment patterns analysed for their band resolution.

Heat generation

During electrophoretic separation the temperatures of conductive media were collected at intervals of 10 min. Our results show that, at high voltage (about 1500 V), the rate of heat generation in TBE medium (90 mM Tris boric acid and 2 mM disodium EDTA) increases over time more rapidly during electrophoresis than in the alkali media tested. The statistical test performed was a one-sample *t*-test using the temperature at 110 min DNA running time for TBE as the null hypothesis against which the mean temperature (at 110 min) from the five new conductive media was compared ($t_4 = 14.7$, $P = 0.0001$). All alkali salts tested could mitigate the positive feedback between current and temperature equally well. Also, the difference in temperature change among the various alkali salt buffers is insignificant (Fig. 1a).

Electrolyte depletion

By plotting the current intensity as a function of time, the potential being relatively constant (but not under control), we estimated the rate of electrolyte exhaustion for each medium tested. The depletion of electrolytes in TBE medium during electrophoresis increased faster than for any of the

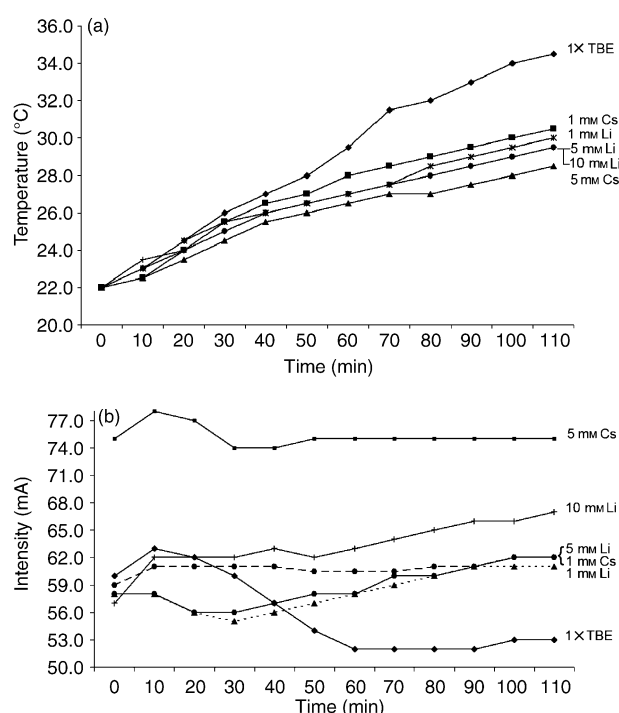


Fig. 1 (a) Rate of heat generation during polyacrylamide electrophoresis at high voltages (about 1500 V). No recirculation or cooling system was applied during the electrophoresis run. (b) Electrolyte exhaustion. Conductive properties of ions in media were tested by measuring current intensity at intervals of 10 min during high voltage electrophoresis runs (about 1500 V).

low concentration conductive media (Fig. 1b). This result is consistent with that of Brody & Kern (2004c), who showed that SB conductive medium (pH 8–9) showed delayed electrolyte exhaustion at constant voltage when compared to TAE and TBE media (3 h for SB compared to 1 h for TAE in agarose gel electrophoresis).

Fragment resolution

Low concentration Li (1 mM) conductive medium that has a low ionic strength gave the best resolution (Fig. 2). In contrast, Tris and 10 mM Li conductive media were responsible for producing unnecessary current, limiting resolution and the ability to run the DNA fragments at a high voltage. Data on the voltage, taken at 10 min intervals during the electrophoresis run, indicate that the more the potential varies (in parallel with the current flow), the lower the resulting fragment resolution (Fig. 2). Ironically, low concentration conductive media do not necessarily exhaust faster as the decrease in current compensates for the reduction in the ionic reserves (Brody & Kern 2004c).

The different resolving abilities of the different conductive media are a consequence of ion solvation (Lopez Cascales & Garcia de la Torre 1997). Ions solvated in aqueous

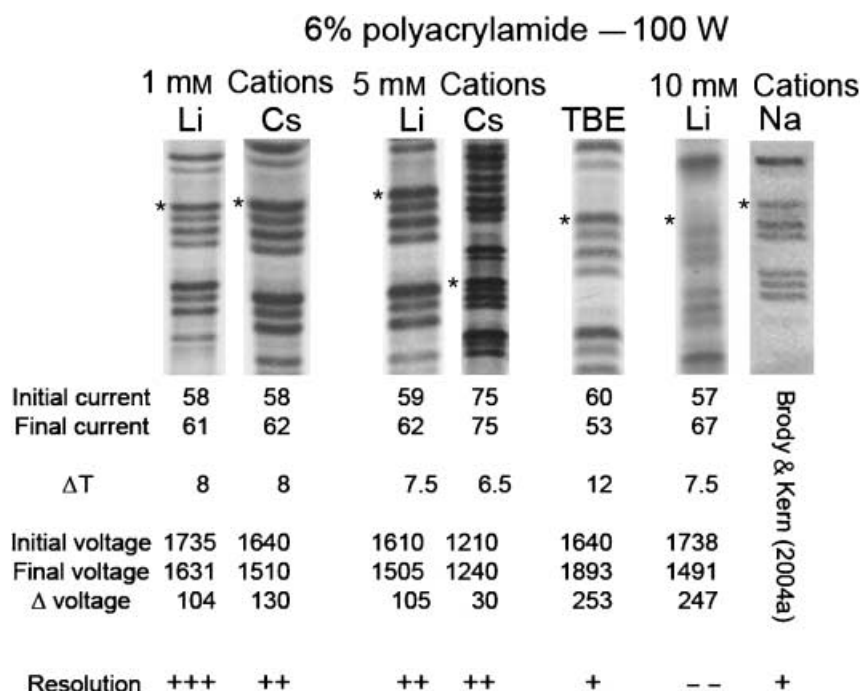


Fig. 2 Separation of microsatellite size-markers using low molarity alkali salt conductive media in 6% denaturing polyacrylamide gel electrophoresis at high voltages (about 1500 V). ΔT indicates the difference between the initial and the final temperature in the media after electrophoresis. Only few fragments from the cytosine–thymine sequence ladder are shown to evaluate the DNA resolution from the different conductive media tested. The stars indicate the location of one identical fragment (121 bp) on each gel after the electrophoretic migration using the various conductive media. An equal amount of DNA (c. 600 ng) was loaded onto each gel. The exposure time of the films to the radioactive fragments was about the same except for the fragments separated in the 5 mM Cs medium which were left 2 days longer and show a stronger signal/intensity than the others. Power was fixed at 100 W. The results show that the commonly used TBE medium separate DNA with a medium resolution (+). Resolution is excellent (+++) for DNA fragments separated in 1 mM Li medium. Resolution is poor (–) for DNA fragments separated in 10 mM Li. The fragment resolution patterns reveal either tight and well separated bands (see 1 mM Li), this pattern suggests a constant charge at the surface of the polyelectrolytes during the electrophoresis run, or broad and overlapped bands/peaks (see 10 mM Li), this pattern suggests a high charge variation at the surface of the polyelectrolytes during the electrophoresis run.

solution are surrounded by a shell of solvent and sometimes by other ions (Loeffler & Rode 2002). If two different ions are of similar charge but one has a larger solvated size, the latter will be less electromobile and less conductive (Brody & Kern 2004c). Lithium has a larger shell of hydration than does sodium, which in turn has a larger hydration shell than does caesium (Karpov 2005). The large hydration shell of Lithium is correlated to its high ionization potential (ratio of the ionic charge to the diameter of the ion) which tends to stabilize the charge variations at the surface of negatively charged DNA molecules and hence the potential measured during the electrophoresis run. Ionic strength is a function of ionic concentration and includes effects of ionic charge. High ionic strength produces a high variation in the potential during the electrophoresis run which could suggest a high charge variation at the surface of DNA molecules, leading to poor resolution with overlaps among DNA fragments (Fig. 2). Also, high ionic strength solutions (e.g. TBE) generated unnecessary heat, which may be an additional factor that could explain the poor band resolution associated with those buffers (Fig. 2).

Conclusion

Low concentration and conductivity solutions of alkali salts used for electrophoretic separation in this study reduced heat generation, permitting much higher voltages (200 v/cm) to be used in gel electrophoresis, thereby decreasing separation times, and enhancing fragment resolution. At high voltage (200 v/cm), 1 mM Li conductive solution showed the best resolution, which is consistent with the large hydration shell of this cation. Overall, we found that the use of low molarity conductive media (e.g. 1 mM Li) reduced the material costs of buffers 10-fold, allowed the elevation of the initial electric field across the sample resulting in a 25% (1.5 h instead of 2 h) time saving per run, and resulted in better DNA fragment resolution.

Acknowledgements

This work was supported by University of Canterbury Masters scholarship, Christchurch, New Zealand. Thank you to Margaret Will (University of Canterbury, Christchurch, NZ) who helped to

complete this study and Dr Guy J. Forrester (Landcare Research, Lincoln, NZ) for his guidance on statistical points.

References

- Aaij C, Borst P (1972) The gel electrophoresis of DNA. *Biochimica Biophysica Acta*, **269**, 192–200.
- Brody JR, Calhoun ES, Gallmeier E, Creavalle TD, Kern SE (2004a) Ultra-fast high-resolution agarose electrophoresis of DNA and RNA using low-molarity conductive media. *BioTechniques*, **37**, 598,600,602.
- Brody JR, Kern SE (2004b) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *BioTechniques*, **36**, 214–216.
- Brody JR, Kern SE (2004c) History and principles of conductive media for standard DNA electrophoresis. *Analytical Biochemistry*, **333**, 1–13.
- Grabar P, Williams CA (1953) Method permitting the combined study of the electrophoretic and the immunochemical properties of protein mixtures; application to blood serum. *Biochimica Biophysica Acta*, **10**, 193–194.
- Karpov GV (2005) On the structure of hydration shells of alkali metal ions in aqueous solutions. *Chemical Physics Letters*, **402**, 300–305.
- Loeffler HH, Rode BM (2002) The hydration structure of the lithium ion. *Journal of Chemical Physics*, **117**, 110–117.
- Lopez Cascales JJ, Garcia de la Torre J (1997) Effect of lithium and sodium ions on a charged membrane of dipalmitoylphosphatidylserine: a study by molecular dynamics simulation. *Biochimica Biophysica Acta*, **1330**, 145–156.
- Sanger F, Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*, **94**, 441–448.
- Sanger F, Nicklen S, Coulson AR (1992) DNA sequencing with chain-terminating inhibitors 1977. *Biotechnology*, **24**, 104–108.
- Tiselius A (1937) A new apparatus for electrophoretic analysis of colloidal mixtures. *Transactions of the Faraday Society*, **33**, 524–531.

HAIR SAMPLING AND GENOTYPING FROM HAIR FOLLICLES: A MINIMALLY-INVASIVE ALTERNATIVE FOR GENETICS STUDIES IN SMALL, MOBILE PINNIPEDS AND OTHER MAMMALS

ABIGAIL K. CAUDRON

Behavioural Biology Unit,
Zoology Institute, University of Liège,
22 Quai Van Beneden,
4020 Liège, Belgium
and

Molecular Ecology, School of Biological Sciences,
University of Canterbury, Private Bag 4800,
Christchurch, New Zealand
E-mail: a.caudron@gmail.com

SANDRA S. NEGRO

CHRISTOPHER G. MULLER

LAURA J. BOREN

NEIL J. GEMMELL

Molecular Ecology, School of Biological Sciences,
University of Canterbury, Private Bag 4800,
Christchurch, New Zealand

Capturing wild mammals for biopsies for DNA analyses can be difficult to achieve, time consuming, and highly stressful for the study animal. Many non- and minimally invasive sampling methods have been tested over the past decades to overcome this problem. Noninvasive methods *sensu* Taberlet *et al.* (1999) imply the collection of biological samples, such as feces, shed hair, and sloughed skin, without the study animal being aware of the collection (Whitehead *et al.* 1990, Amos *et al.* 1992, Clapham *et al.* 1993, Reed *et al.* 1997). However, noninvasive sampling is prone to systematic genotyping errors due to the small amount of DNA collected, the uncertainty regarding the identity of the individual from which the DNA comes, cross-contamination among individuals, and the degradation of samples exposed for an unknown period of time (*e.g.*, Pemberton *et al.* 1995, Taberlet *et al.* 1996, Gagneux *et al.* 1997, Taberlet *et al.* 1999, Hoffman and Amos 2005). By their nature, noninvasive methods can also be time-consuming and frustrating if researchers have to wait for a target animal to produce a sample, if indeed a sample can ever be obtained.

Minimally invasive approaches are defined as intermediate, in terms of impacts on the animal, between capture for biopsy and noninvasive sampling. By selectively sampling an individual from a distance and obtaining a sufficient amount of fresh material for DNA analyses, most of the drawbacks of both the fully invasive and the

noninvasive methods can be avoided. For more than 30 yr, cetacean skin samples have been successfully collected with modified rifles, crossbows, or archery, using projectiles fitted with a biopsy core (Winn *et al.* 1973, Lambertson 1987, Barrett-Lennard *et al.* 1996). The bow riding behavior displayed by many small cetaceans has also been exploited for skin collection using a scraping sponge mounted on a pole (Harlin *et al.* 1999).

In pinnipeds, DNA sampling has been successfully achieved by collecting feces, by collecting skin samples using a modified crossbow (fur seals: Gemmell and Majluf 1997, gray seals: Worthington-Wilmer *et al.* 1999, walruses: Wiig *et al.* 2000), or during capture. No study specifically investigated and compared the disturbance these methods cause to seals. However, we postulate that invasiveness increases notably with the increasing duration of the sampling operation, with the increasing number of humans involved in the operation, and with the decreasing sampling distance, capture being a climax. Fecal samples have been used for the genotypic identification of species (Reed *et al.* 1997), but collecting feces is not suited to many studies of behavior because seals' gut transit times can be very slow, especially as they fast in the breeding season. Skin sampling using a crossbow is not advisable for certain categories of pinnipeds (small, mobile, skittish) where this is potentially dangerous for both the sampled seal and the neighboring seals. Fresh hair sampling from target individuals can provide a useful compromise between noninvasive techniques and capture. Hair follicles as a source of DNA have been used in many species (for references, see Section 1 of the Electronic Appendix for this paper). However, there is as yet no published information on the use of hair samples for genotyping pinnipeds or any other aquatic mammal.

For an ongoing study of paternity in the New Zealand fur seal, *Arctocephalus forsteri*, we needed to genotype mother-pup pairs in order to match them with putative fathers. Males were skin sampled with a crossbow (Gemmell and Majluf 1997) while pups were captured and a small skin sample was collected from their flipper with piglet ear notch pliers (Majluf and Goebel 1992). However, adult females could not be approached easily (skittish and surrounded by aggressive males) and we considered it too risky for the animals to attempt skin sampling (skin puncture) on such mobile and small targets (females 30–60 kg *vs.* males 180–200 kg, Reeves *et al.* 1992). Crossbow biopsies from large male fur seals (with a thicker blubber than in females) showed that the cores sometimes penetrate up to the muscle layer. In addition, most of our study females were not marked and were only identifiable when interacting with their marked pup, which added the extra risk of injuring the pup. Collecting shed hair was not an option, because we required fresh samples from known individuals in a large colony. Consequently, we had firstly, to adapt an existing method (crossbow skin biopsy) to sample seal hair without skin puncture, and secondly, to design an adequate genotyping protocol from seal hair follicles.

The study site was Ohau Point fur seal breeding colony (approximately 2,200 seals), 25 km north of Kaikoura (42°25'0"S, 173°40'60"E), New Zealand, during the 2003–2004 breeding season. A modified crossbow (36.3 kg pull Steelforce, Horton, Manufacturing Co. Inc., Tallmadge, OH) was used as described by Gemmell and Majluf (1997) with custom-designed bolt heads filled with adhesives, to collect hairs

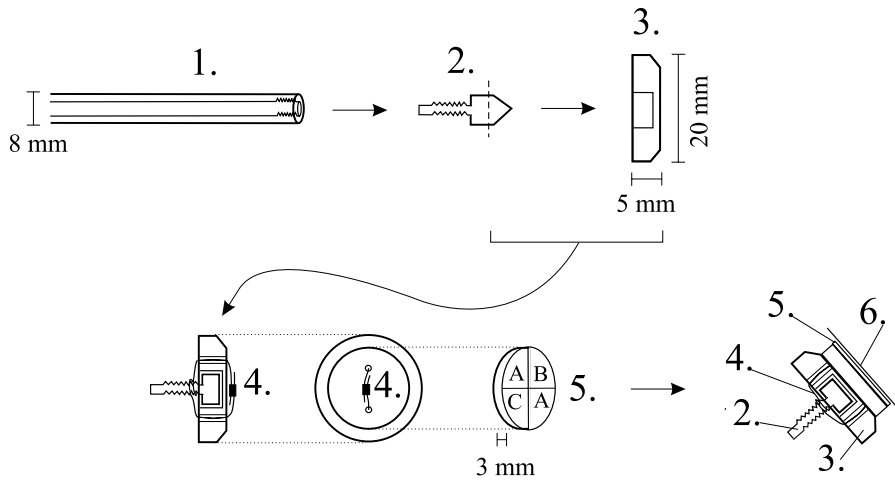


Figure 1. Bolt heads for hair sampling. Top: 1. bolt shaft, 2. original bolt head with screw thread, 3. PVC disk, 4. metal trace to prevent the loss of the disk, 5. neoprene disk fitted with adhesives of types A, B, and C (thin glue layer on a translucent plastic support, Aeroxon Cockroaches Traps). Bottom: complete head with film protecting the adhesive (6).

instead of skin cores (Fig. 1). A sampling jab-stick with removable heads was also made for close-range collection, which was ideal for occasional cases when a target female could be approached within 2 m, for example, when it was asleep behind a rock. Finding an adequate adhesive was critical, because fur seal coats are very dense, slightly oily, and often wet. The bolt heads and jab-stick designs are described in Section 2 of the Electronic Appendix, together with the range of adhesives we trialed.

Heads were used only once for one individual, then sanded clean, and a new neoprene disk with adhesives was attached. Pulled hairs were stored in a vial containing 70% ethanol, by cutting through the thickness of the neoprene disk and placing the whole sticky pad with attached hairs inside the vial. Under laboratory conditions, follicles (clearly visible on a dark background) were counted, cut (maximum 3 mm of length was kept, including the follicle) and transferred into a smaller vial (70% ethanol) for storage at -20°C until genotyping. All sampling equipment was flamed with ethanol between samples.

DNA was extracted from skin biopsies (positive controls) with a method modified from Walsh *et al.* (1991). But we had to design a new protocol in order to maximize DNA extraction from hair (described in Section 3 of the Electronic Appendix). To amplify DNA fragments through polymerase chain reaction (PCR), we used previously described methods (Gemmell *et al.* 1997, Davis *et al.* 2002) but optimized the process by slightly increasing MgCl_2 (2 mM MgCl_2) and Taq Polymerase concentrations (0.25 units/10 μL reaction). Highly specific DNA fragments were amplified from a small amount of material with a high titer through a Touchdown PCR that ranged from 70°C to 40°C (Don *et al.* 1991). PCR details can be found in Section

Table 1. Details of hair sampling trials using the crossbow with modified bolt heads and the jab-stick.

Tool used	Number of trials	Hits	Samples (≥ 3 hairs)
Crossbow	75 (86% of all trials)	58 (77% of crossbow trials)	43 (74% of crossbow hits)
Jab-stick	12 (14% of all trials)	8 (67% of jab-stick trials)	8 (100% of jab-stick hits)
Total	87, on a total of 60 target females	66 (76% of all trials)	51 samples (but 2 for the same target female, so 50 individuals sampled in total)

4 of the Electronic Appendix. We quantified the amount of DNA extracted from increasing numbers of hair follicles using a UV-visible spectrophotometer (Shimadzu UV-1601 PC, Shimadzu Corp., Kyoto, Japan).

From a total of 87 sampling trials (including 4 trials where the jab-stick was used as a second choice, after a missed crossbow shot) on a total of 60 individuals, hair samples were successfully collected from 50 lactating female New Zealand fur seals (Table 1). Out of the 50 seals successfully sampled, 85% ($n = 43$) were sampled with the crossbow and 15% ($n = 8$) with the jab-stick. Samples ranged from 3 to over 200 guard hair follicles ($\bar{x} = 68 \pm 58$ follicles, median = 55, inter-quartile interval = 11–109, $n = 51$ samples). The accuracy (total hitting success) was 76% (66 hits out of 87 trials, pooling the crossbow and the jab-stick), and the sampling success (≥ 3 follicles) was 77% of all hits (15 hits out of 66 did not provide a sample). The jab-stick was rarely suitable as a first choice method (9% of all trials), but when it was, its hitting and sampling success were 100%, suggesting an excellent potential for that tool.

Our goal was to collect samples, so conditions that did not work were avoided as soon as they were identified, instead of specifically investigated and quantified. However, four major impeding factors could be identified: the distance to the target, the ambient air temperature, bolts failures, and the skittishness of the target.

Firstly, when a seal was hit, the chances of getting a sample increased if the shooter approached as close as possible, undetected by the target. We considered 4 m as the closest safe distance for crossbow shooting; at closer range, sampling with the jab-stick was a better option. The modified heads did not unduly impede the bolt's flight, but the bolts tended to drop quickly over 10 m. A skilled shooter could usually compensate for the trajectory drop at long range, but the bolt often lacked enough momentum to stick to the hair. The maximum range for a successful crossbow hit with the modified sample heads was approximately 15 m.

Secondly, increasing temperature increased the adhesive properties. It also most probably influenced the strength required to remove hair (skin constriction when colder). To accommodate uncertainties due to differences in ambient temperature, we designed sticky pads with portions of adhesives A (a thin glue layer on a plastic support, SKP-10 Replacement Insect Sticky Pads, Starkeys Products, Perth, Australia), B (thick glue layer on a cardboard support, Protecta MC Glue Trap for Insects and Mice, Bell Laboratories, Inc., Madison, WI), and C (thin glue layer on a translucent

plastic support, Aeroxon Cockroaches Traps, Aeroxon Insect Control GmbH, Waiblingen, Germany), so that there would always be a portion likely to catch hair in the operational air temperature range of 10°–30°C (Fig. 1). It was critical to thin the backing layer of the adhesive material to a minimum to avoid a weak point where the layers could separate (especially in the case of adhesive B), leaving the actual adhesive pad stuck on the seal instead of pulling hair back with the bolt/stick. Out of the 15 hits that provided no samples, 9 (60%) were due to the disk not sticking because the part of the coat hit was damp, because it had hair too short, or because the air temperature was too cold, reducing the stickiness of the adhesives. We recommend avoiding damp portions of the target's coat, targeting areas of long guard hair (neck, chest, back), and ensuring an impact angle as close as possible to 90° with the surface of the seal's body to maximize the contact area of the adhesive pad.

In 3 of the 15 hits without samples, the sample was lost due to equipment failure, *i.e.*, the bolt head broke off or the fishing line broke (firing stress and/or worn on rocks). Solutions included using a piece of metal trace to secure the disk on the bolt (Fig. 1), and using a split ring and a fishing-line swivel at the bolt/line junction to minimize the tendency of the line to snap.

Finally, seals' skittishness clearly varied with breeding status. The colony was far less skittish during the November–January breeding season than later in the austral summer. The increased skittishness of the targets over the season countered the increased experience of the shooter, so the proportion of missed shots did not decrease over time (12.5% in February, 22.6% in March, 21.4% in April). In 59% of all the missed shots, the target female moved out of range as the shot was taken, either due to her natural behavior or being alerted by the shooter's approach (although moving targets provided 4% of the successful samples, provided the shooter was within range at the time). In addition, skittishness was affected by previous disturbance on the sampling day; therefore it was ideal to alternate sampling days between sites. It was also affected by the social surrounding of the target female (harder to approach if she was in a dense group of seals). The topography (rocks or platform) affected hiding spots and likely approach for the shooter. The present study did not focus on nor quantified the response of the targets, as all of them reacted to both hits and close misses: most sampled seals fled toward the sea. However, if the two people collecting samples were not spotted, the target often remained, or only ran a short distance and stood up alert, looking around. Individuals were more skittish after having been hit in the past. All targets were seen back interacting with their pup at their usual resting spot, either within a few hours or within a few days (after a feeding trip at sea).

DNA extraction was performed from varying numbers of follicles from one individual, using the same protocol ($n = 13$ vials, with, respectively, 2–6, 8, 10, 12, 14, 16, 20, 24, 30 follicles per vial). A skin biopsy from the same individual was used as positive control to check for PCR contaminations, misamplification, and erroneous DNA separation (*i.e.*, electrophoresis). The amount of DNA recovered from our extraction protocol increased as a function of the number of hair follicles extracted (multiple $r^2 = 0.806$, Fig. 2A). However, the amount of amplified product from the PCR was already sufficient for analysis when only a very small number of follicles

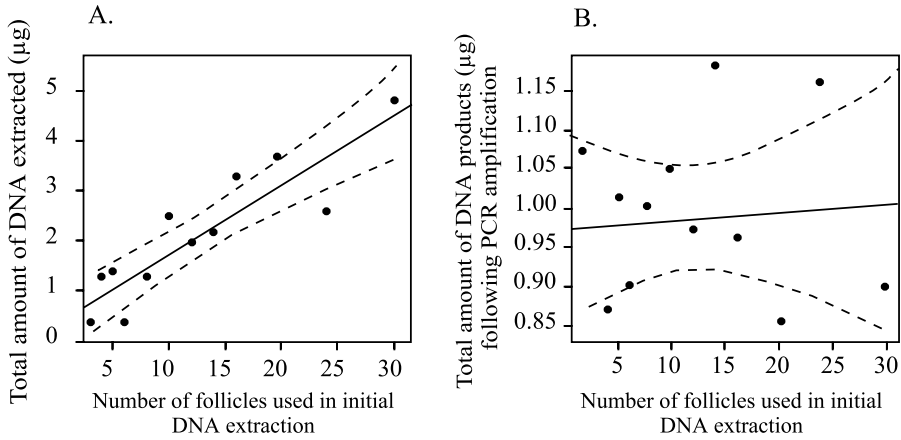


Figure 2. (A) The amount of DNA (μg) recovered during extraction increases with an increasing number of fur seal hair follicles. (B) Amplification through Touchdown PCR generates enough DNA for genotypic analysis, even from extracts of a low number of follicles. Dotted lines are multiple regressions with 95% confidence intervals.

($n = 3$) were used in the original extraction (Fig. 2B). Amplification of 14 microsatellite markers was achieved: for each one of the 13 vials, 3 triplicates were amplified, run on a gel, and scored. Two additional controls from other individuals with known genotypes were used. We estimated the reliability of our DNA extraction and PCR methods by calculating the microsatellite locus-specific and overall genotyping error rates (Table 2).

Table 2. Estimation of the genotyping error rates per reaction and per allele for the current study. The number of reactions differs among loci due to a small number of missing single-locus genotypes.

Locus	No. of reactions	No. of mistyped reactions	No. of mistyped alleles	Error rate per reaction	Error rate per allele
Hg4.2	33	0	0	0	0
Hg8.10	37	1	1	0.027	0.014
Hg1.4	33	0	0	0	0
Lc28	33	0	0	0	0
HI16	34	0	0	0	0
Pv9	32	0	0	0	0
PvcA	37	0	0	0	0
Pv11	39	1	1	0.026	0.013
G1A	32	0	0	0	0
Hg6.3	34	0	0	0	0
M11 A	37	0	0	0	0
3E3	37	1	1	0.027	0.014
Lc5	32	0	0	0	0
Hg 6.1	33	0	0	0	0
Overall	553	3	3	0.005	0.003

Scoring errors represented the majority of genotyping errors for DNA extracted from c. 10 hair follicles. But for extractions from fewer follicles, the majority of errors were due to allelic dropout. The error rate per reaction was calculated as the number of incorrect genotypes divided by the total number of reactions (Hoffman and Amos 2005). Likewise, the error rate per allele was calculated as the number of incorrect alleles divided by the total number of alleles. An allele was recorded as being mistyped when a difference was observed between three genotypes. Errors were observed at Hg 8.10, Pv11, and 3E3 with recorded error rates per alleles ranging from 0.013 to 0.014.

Obtaining permits for captures and biopsies in pinnipeds is becoming increasingly difficult (Viggers 2000, Widolf 2002) and hair sampling provides a more readily acceptable alternative to many of the current sampling strategies. The disturbance of our sampling approach to the target seal and to the surrounding portion of the colony was not negligible as the target was aware of the sampling and there was a human intrusion in the colony. It was however obviously less disruptive than capturing a target seal for a skin biopsy where the target is chased, netted, and restrained, the human intrusion in the colony is larger and lasts longer. The factors impeding sampling were similar to those identified by Gemmell and Majluf (1997) for skin biopsies with a crossbow. It required experienced field biologists with at least a few weeks' experience of working (moving) through a fur seal breeding colony and the shooter being familiar with using a crossbow, and practicing both before and in-between sampling days. Some additional impeding factors were specific to hair collection, such as the dampness of the coat and ambient air temperature.

Our hair sampling and genotyping protocols for pinnipeds enables the collection of genetic data with a reduced risk of injuring the target animal or a nearby pup, and with an overall genotyping error rate of *ca.* 0.005 per reaction. With the need for only three or more hairs, it is probable that this approach could be utilized on any mammal of moderate size.

Although we did not fully test its potential, the additional method we present (the jab-stick) tended to provide better samples (more hair) than the crossbow. It is an ideal tool for hair sampling at close range, for example, from territorial males and aggressive mothers earlier in the fur seal breeding season. The jab-stick also offers an alternative for captive mammals that are difficult to restrain or on which immobilization is risky, due to its ability to be passed through the bars of a cage, for example.

ACKNOWLEDGMENTS

Fieldwork was based at the Edward Percival Field Station, University of Canterbury (UC), New Zealand. J. Van Berkel helped to manufacture the bolt heads. Dr. B. Robertson provided some preliminary observations regarding bolt modifications. AC's postdoc was supported by the Belgian FNRS, King Léopold III Fund for Nature Exploration and Conservation, and the Duesberg Fund (University of Liège, Belgium). SN's genetics work was supported by a UC Master's Scholarship. Hair and tissue samples were collected under the permit Per/10/2002/01, DOC, NZ. We thank Dr. Sean Twiss and an anonymous referee for their helpful comments on the manuscript.

LITERATURE CITED

- AMOS, W., H. WHITEHEAD, M. J. FERRARI, D. A. GLOCKNER-FERRARI, R. PAYNE AND J. GORDON. 1992. Restrictable DNA from sloughed cetacean skin; its potential for use in population analysis. *Marine Mammal Science* 8:275–283.
- BARRETT-LENNARD, L. G., T. G. SMITH AND G. M. ELLIS. 1996. A cetacean biopsy system using lightweight pneumatic darts, and its effect on the behavior of killer whales. *Marine Mammal Science* 12:14–27.
- CLAPHAM, P. J., P. J. PALSBOØLL AND D. K. MATTILA. 1993. High-energy behaviors in humpback whales as a source of sloughed skin for molecular analysis. *Marine Mammal Science* 9:213–220.
- DAVIS, C. S., T. S. GELATT, D. SINIFF AND A. STROBECK. 2002. Dinucleotide microsatellite markers from the Antarctic seals and their use in other Pinnipeds. *Molecular Ecology Notes* 2:203–208.
- DON, R. H., P. T. COX, K., B. J. WAINWRIGHT, K. BAKER AND J. S. MATTICK. 1991. “Touchdown” PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* 19:4008.
- GAGNEUX, P., C. BOESCH AND D. S. WOODRUFF. 1997. Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology* 6:861–868.
- GEMMELL, N. J., AND P. MAJLUF. 1997. Projectile biopsy sampling of fur seals. *Marine Mammal Science* 13:512–516.
- GEMMELL, N. J., P. J. ALLEN, S. J. GOODMAN AND J. Z. REED. 1997. Interspecific microsatellite markers for the study of pinniped populations. *Molecular Ecology* 6:661–666.
- HARLIN, A. D., B. WURSIG, C. S. BAKER AND T. M. MARKOWITZ. 1999. Skin swabbing for genetic analysis: Application to dusky dolphins (*Lagenorhynchus obscurus*). *Marine Mammal Science* 15:409–425.
- HOFFMAN, J. I., AND W. AMOS. 2005. Microsatellite genotyping errors: Detection approaches, common sources and consequences for paternal exclusion. *Molecular Ecology* 14:599–612.
- LAMBERTSON, R. 1987. A biopsy system for large whales and its use for cytogenetics. *Journal of Mammalogy* 68:443–445.
- MAJLUF, P., AND M. GOEBEL. 1992. The capture and handling of female South American fur seals and their pups. *Marine Mammal Science* 8:187–190.
- PEMBERTON, J. M., J. SLATE, D. R. BANCROFT AND J. A. BARRETT. 1995. Nonamplifying alleles at microsatellite loci: A caution for parentage and population studies. *Molecular Ecology* 4:249–252.
- REED, J., D. J. TOLLIT, P. M. THOMPSON AND W. AMOS. 1997. Molecular scatology: The use of molecular genetic analysis to assign species, sex and individual identity to seals. *Molecular Ecology* 6:225–234.
- REEVES, R. R., B. S. STEWART AND S. LEATHERWOOD. 1992. The Sierra Club handbook of seals and sirenians. Sierra Club Books, San Francisco, CA.
- TABERLET, P., S. GRIFFIN, B. GOOSSENS, S. QUESTIAU, V. MANCEAU, N. ESCARAVAGE, L. P. WAITS AND J. BOUVET. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* 24:3189–3194.
- TABERLET, P., L. P. WAITS AND G. LUIKART. 1999. Noninvasive genetic sampling: Look before you leap. *Trends in Ecology and Evolution* 14:323–327.
- VIGGERS, K. 2000. Seals saga – data needed. *Australian Veterinary Journal* 78:525–526.
- WALSH, P. S., D. A. METZGER AND R. HIGUCHI. 1991. Chelex 100 as a medium for the simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10:506–513.
- WHITEHEAD, H., J. GORDON, E. A. MATHEWS AND K. R. RICHARD. 1990. Obtaining skin samples from living sperm whales. *Marine Mammal Science* 6:316–326.

- WIDOLF, H. E. 2002. Animal rights and the Antarctic treaty system. *Human Ecology Review* 9:59–67.
- WIIG, Ø., V. BERG, I. GJERTZ, D. J. SEAGARS AND J. U. SKAARE. 2000. Use of skin biopsies for assessing levels of organochlorines in walruses (*Odobenus rosmarus*). *Polar Biology* 23:272–278.
- WINN, H. E., W. L. BISHOFF AND A. G. TARUSKI. 1973. Cytological sexing of Cetacea. *Marine Biology* 23:343–346.
- WORTHINGTON-WILMER, J., P. J. ALLEN, P. P. POMEROY, S. D. TWISS AND W. AMOS. 1999. Where have all the fathers gone? An extensive microsatellite analysis of paternity in the grey seal (*Halicboerus grypus*). *Molecular Ecology* 8:1417–1430.

Received: 20 June 2005

Accepted: 6 June 2006

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:
Supplementary Appendix and Additional References

Appendix 1 -- technical details and extra references related to the manuscript

Caudron, A. K., S. S. Negro, C. G. Muller, L. J. Boren and N. J. Gemmell. 2006. Hair sampling and genotyping from hair follicles : A minimally-invasive alternative for genetics studies in small, mobile pinnipeds and other mammals.

Section 1: Literature - Hair follicles have been used as source of DNA for genetics studies notably in ursids, felids, mustelids, canids: Raphael 1994, Foran *et al.* 1997, Taberlet *et al.* 1997, Valderrama *et al.* 1999, Woods *et al.* 1999, Mowat and Paetkau 2001, Paetkau 2003, Creel *et al.* 2003 ; elephants : Greenwood and Pääbo 1999 ; primates : Clifford *et al.* 1999, Valderrama *et al.* 1999, Constable *et al.* 2001, Morin *et al.* 1993 ; marsupials: Sloane *et al.* 2000 ; review : Taberlet *et al.* 1999.

Section 2: Bolt heads and jab-stick design - Fur seal coats are very dense (465 hairs/mm², Riedman 1990), slightly oily and often wet, so finding an adequate material for adhering to and removing hair was critical. We trialled a number of sticky materials previously reported in the literature for sampling hair in other mammals (mouse/rat glue trays : Foran *et al.* 1997, sticky tape : Sloane *et al.* 2000 ; liquid mouse glue: Mowat and Paetkau 2001) as well as a variety of other adhesive products *e.g.*, sports plaster, waterproof insulation tape, cosmetic hair removal wax, fly-catching ribbon, rat glue boards, spray-on craft glue, insect monitoring sticky pads. Adhesives were trialled on a freshly dead fur seal carcass found on the beach. Materials that successfully extracted coarse guard hairs with their follicle from a fresh seal carcass found on the beach were then trialled by hand on a live female fur seal already restrained for tagging. This single capture also allowed a skin biopsy to be taken, as a positive control for subsequent genotyping tests. Finally, we used a combination of three adhesives, based on their performance during initial trials (Figure 1).

We custom-designed bolt heads: the removable tips of crossbow target bolt heads (Horton, USA) were sawn flat (Figure 1: 2.) and each was housed into a matching recess drilled at the center of a 20 mm diameter PVC disk (3.). The hole was not drilled right through the disk; 2-3 mm of plastic remained, ensuring the bolt shaft could not pass through the disk at the moment of impact. This ensured the impact force was spread over the entire disk, preventing the bolt's penetration. The front edge of the PVC disk was beveled to present a rounder surface, and therefore allow a slightly wider angle of successful shots. The bolt head's original screw thread remained, allowing the disk to be rapidly attached and detached from an bolt. A weak point where the screw was glued into the PVC disk was intentional, so that when a missed shot hit a solid surface (highly likely in rocky terrain), the PVC disk would break off or be damaged, rather than the shaft (more costly). However, to avoid losing heads along with samples, a piece of metal fishing-trace (4.) was passed through 2 fine holes in the disk (as in a button) and attached to the base of the screw thread. This still allowed the head to break at this point if hitting rocks, but ensured it was not lost along with the sample. Araldite 2-part epoxy resin was used to glue the hair-collecting adhesive to a circle of neoprene providing padding (5.), and to glue the neoprene onto the PVC disk. To be retrieved after a successful shot, the bolt was attached to a fishing line and reel mounted to the crossbow (Gemmell and Majluf 1997).

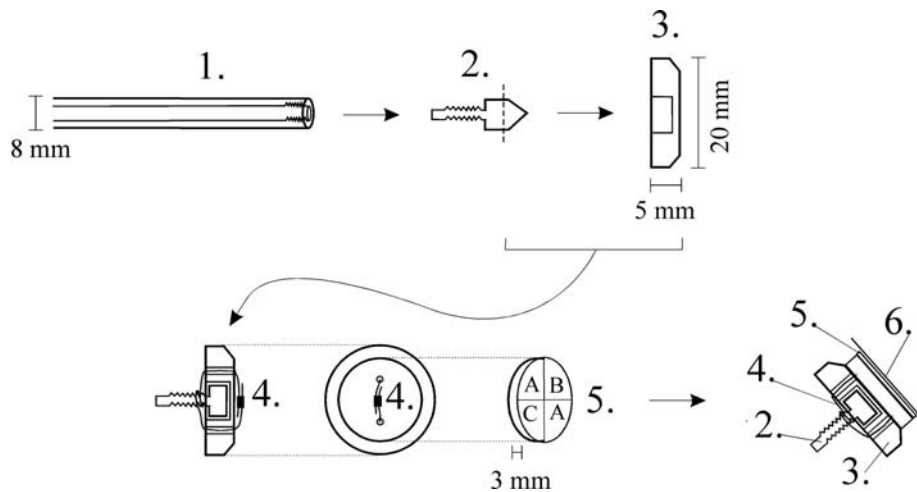


Figure 1. Bolt heads for hair sampling: Top: 1. bolt shaft. 2. original bolt head with screw thread. 3. PVC disk. 4. metal trace to prevent the loss of the disk. 5. neoprene disk fitted with adhesives of types A (thin glue layer on a plastic support, SKP-10 Replacement Insect Sticky Pads, Starkeys Products, Australia), B (thick glue layer on a cardboard support, Protecta MC Glue Trap for Insects and Mice, Bell Laboratories, Inc.) and C (thin glue layer on a translucent plastic support, Aeroxon Cockroaches Traps). Bottom: complete head with film protecting the adhesive (6.)

The jab-stick was made from a 2 m long wood stick with a 3 cm diameter. Removable heads were made from 20 cm long portions of a PVC pipe. The pipe sections had an adequate diameter to fit tightly at the end of the pole, but could be twisted off. Pipe sections were closed with a 3 mm thick disk (wood or PVC) glued on the pipe's front edge. A 3 mm thick neoprene disk (padding) with a combination of adhesives A, B and C was glued (2-part epoxy resin) in the same manner as the bolt heads were made. Two or 3 spare heads were brought on each sampling day.

Section 3: DNA Extraction - Whole genomic DNA was extracted from hair as follows. Ten follicles were put into 100 μ l Chelex 100 resin (5% Chelex in 10 mM Tris, 0.1 mM EDTA). The endolytic protease, proteinase K (1.4 μ l 20 mg/ml) and the reducing agent, dithiothreitol (2.8 μ l 1M DTT), were then added to the solution. The sample was then incubated at 58 °C for 2 hours with occasional shaking, followed by a second addition of Proteinase K (1.4 μ l 20 mg/ml) and further incubations at 58 °C for 2 hours. Next, the solution was boiled for 8 min, vortexed for c. 15 s, centrifuged at 20,800 x g for 4 min, and the resulting supernatant transferred to a new vial and stored at -20 °C.

Section 4: DNA amplification - The PCR was carried out in 10 μ l reaction containing c. 30 ng of template DNA, 0.5 pmol of each primer, 5 nmol each of dATP, dGTP, and dTTP, 0.5 nmol dCTP, 0.1 μ Ci of α P³³-dCTP, 50 mM KCl, 10 mM Tris-HCl at pH 9.0, 2 mM MgCl₂ and 0.25 unit of Taq polymerase (Biolabs, New England). A Touchdown PCR was undertaken in which the annealing temperature was reduced every two cycles from 70 to 50 °C, 4 cycles at 45°C, and 28 cycles at 40°C. The general thermocycling profile for all loci was: 94°C/30s, 70-40°C/30s and 72°C/45s. This thermocycling profile resulted in specific and high titer amplification of the target fragments. PCR products were size-fractionated on 6% denaturing polyacrylamide gels using a conductive medium containing a low concentration of alkali salts (Negro *et al.* 2005). After gel drying, the amplified fragments were exposed for 3 days on Kodak Biomax MR autoradiography film (Radiographic Supplies, Christchurch, NZ) and the size of the amplified fragments scored manually against a sequencing reaction (forward M13 primer and pBSMB plasmid).

Additional references:

- Clifford, S. L., K. Jeffrey, M. W. Bruford, and E. J. Wickings. 1999. Identification of polymorphic microsatellite loci in the gorilla (*Gorilla gorilla gorilla*) using human primers: application to non invasively collected hair samples. *Molecular Ecology* 8:1551-1561.
- Constable, J. L., M. V. Ashley, J. Goodall, and A. E. Pusey. 2001. Noninvasive paternity assignment in Gombe chimpanzees. *Molecular Ecology* 10:1279-1300.
- Creel, S., G. Spong, J. L. Sands, J. Rotella, J. Zeigle, L. Joe, K. M. Murphy, and D. Smith. 2003. Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Molecular Ecology* 12:2003-2009.
- Foran, D. R., S. C. Minta, and K. S. Heinemeyer. 1997. DNA-based analysis of hair to identify species and individuals for population research and monitoring. *Wildlife Society Bulletin* 25:840-847.
- Gemmell, N. J., and P. Majluf. 1997. Projectile biopsy sampling of fur seals. *Marine Mammal Science* 13:512-516.
- Greenwood, A.D., and S. Pääbo. 1999. Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. *Molecular Ecology* 8:133-137.
- Morin, P. A., J. Wallis, J. J. Moore, R. Chakraborty, and D. S. Woodruff. 1993. Non-invasive sampling and DNA amplification for paternity exclusion, community structure, and phylogeography in wild chimpanzees. *Primates* 34:347-356.
- Mowat, G., and D. Paetkau. 2001. Estimating marten population size and distribution using hair capture and DNA fingerprinting in the central Selkirk mountains of British Columbia. Rep. Slokan Forest Products, 709 Delaney Ave., Slokan, B.C. and Ministry of Environment, Lands, and Parks, Fish and Wildlife Division Kootenay Region Nelson, B.C. 15.
- Negro, S. S., R. Cloots, and N. J. Gemmell, 2006. Cost-effective media for the rapid high resolution of small DNA fragments using polyacrylamide-based electrophoresis. *Molecular Ecology Notes*. *In press*
- Paetkau, D. 2003. An empirical exploration of data quality in DNA-based population inventories. *Molecular Ecology* 12:1375-1387.
- Raphael, M.G. 1994. Techniques for monitoring populations of fishers and American martens. Pages 224-240 in Martens, sables and fishers : Biology and conservation. S. W. Buskirk, A. S. Harestad, M. G. Raphael, and R. A. Powell eds. Cornell Univ. Press. Ithaca, N.Y.
- Riedman, M. 1990. *Pinnipeds: Seals, Sea Lions, and Walruses*. University of California Press: Berkeley, p 1-149.
- Sloane, M.A., P. Sunnucks, D. Alpers, L. B. Beheregaray, and A. C. Taylor. 2000. Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method. *Molecular Ecology* 9:1233-1240
- Taberlet, P., J.-J. Camarra, S. Griffin, E. Uhrès, O. Hanotte, L. P. Waits, C. Dubois-Paganon, T. Burke, and J. Bouvet. 1997. Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology* 6:869-876.
- Taberlet, P., L. P. Waits, and G. Luikart. 1999. Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution* 14:323-327.
- Valderrama, X., W. B. Karesh, D. E. Wildmanand, and D. J. Melnick. 1999. Noninvasive methods for collecting fresh hair tissue. *Molecular Ecology* 8:1749-1752.
- Woods, J.G., D. Paetkau, D. Lewis, B.N. McLellan, M. Proctor, and C. Strobeck. 1999. Genetic tagging free ranging black and brown bears. *Wildlife Society Bulletin* 27:616-627.

Rearing two New Zealand fur seal (*Arctocephalus forsteri*) pups to weaning

Sacha A. Dowell^A, Laura J. Boren^{A,C}, Sandra S. Negro^A, Chris G. Muller^A,
Abigail K. Caudron^{A,B} and Neil J. Gemmell^A

^ASchool of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

^BBehavioural Biology Unit, University of Liège, 22 Quai Van Beneden, 4020 Liege, Belgium.

^CCorresponding author: Email: seals_n_sealions@yahoo.co.nz

Abstract. The rearing of two pups to weaning is a rare occurrence in pinnipeds and in many cases it remains unknown whether it is a result of twinning or fostering. This study followed two cases where female New Zealand fur seals (*Arctocephalus forsteri*) raised two pups, at a colony in Kaikoura, New Zealand. Maternal attendance behaviour was observed, mass and condition of the pups were measured and genetic samples were collected to assess relatedness. In one case, the female gave birth to twins, while the other case was found to be fostering of a second pup. In both cases, the filial pups of each female exhibited significantly lower mass, condition and growth rates than the colony average. The twins' mother reared both pups to weaning and did not appear to spend a different amount of time ashore compared with single-pup mothers. The current study confirms both fostering and twinning in New Zealand fur seals, with the potential for successfully raising the pups to weaning, despite their well developed recognition system and the energy costs involved.

Introduction

Many species of pinnipeds are highly gregarious breeders, where large numbers of individuals gather in limited pupping space and potentially harsh conditions (Riedman 1982). These situations seem ideal for allosuckling, i.e. the nursing of non-filial pups by females, to occur. Allosuckling in pinnipeds was traditionally thought to be an extremely rare occurrence (Marlow 1972; Stirling 1975). However, when studied both anecdotally and quantitatively, it was found to be more widespread than expected (Boness *et al.* 1992; Arnborn *et al.* 1997; Georges *et al.* 1999). There are two forms of allosuckling: fostering, where a non-filial pup is nursed by a female aware of the pup's presence; and milk stealing, where a female remains unaware of the nursing or rejects the non-filial pup as soon as it is discovered (Lunn 1992).

Fostering has been reported in several phocid species that breed in large colonies but rarely occurs in otariids (Stirling 1975; Trillmich 1981; Riedman 1982; Lunn 1992). Lactation in pinnipeds is costly for mothers (Clutton-Brock *et al.* 1989), more so in otariids owing to their extended period of lactation (Oftedal *et al.* 1987). The nursing of non-filial young should be selected against unless reproductive benefit for the female outweighs the costs of fostering. Some hypotheses to explain the occurrence of fostering include: kin selection, whereby an individual's inclusive fitness is increased (Hamilton 1964; Gemmell 2003), the need to evacuate surplus milk (Roulin 2002) or maintain the concentration of the neurohormone prolactin to enhance immunocompetence (Roulin 2003); or to gain maternal skills, as many inexperienced females who have lost their pup adopt a non-filial pup (Riedman and Le Boeuf 1982; Boness *et al.* 1992; Schaeff *et al.* 1999; Roulin 2002).

However, the more common explanation of fostering is recognition error (Riedman 1982; Roulin 2002). Recognition systems are highly developed in otariids because of the need for females to leave their pups in large, dense colonies and relocate them over an extended period of lactation (Riedman 1990; Insley 2001; Dobson and Jouventin 2003; Phillips 2003).

Twinning is extremely rare in pinnipeds (Spotte 1982). Several cases of twin fetuses have been observed but it appears that twins are rarely born alive (Vania 1965; Bryden 1966; Rae 1969; Spotte 1982; Ling 1986; Fay *et al.* 1991). As a result, some authors assume that a female observed with two pups is fostering one of them (Childerhouse and Gales 2001). On the other hand, several other authors have assumed that females nursing two pups are cases of twinning because fostering was thought not to occur in otariids (Bester and Kerley 1983; Doidge 1987). Molecular methods to determine relatedness provide a more reliable technique than mother–pup associations in order to distinguish between twinning and fostering (Miller 1971; Gelatt *et al.* 2001; McMahon and Hindell 2003).

The success of otariid females rearing two pups to weaning is rare, having been well described in only three species: Antarctic fur seals (*A. gazella*) (Doidge 1987), subantarctic fur seals (*A. tropicalis*) (Bester and Kerley 1983; Georges *et al.* 1999) and *A. forsteri* (Haase 2007). For pups sharing a mother, growth rates were reduced in both Antarctic fur seals (Doidge 1987) and subantarctic fur seals (Georges *et al.* 1999) compared with singletons. Weaning happened later for foster–filial pup dyads than for pups raised singly (Georges *et al.* 1999; Haase 2007). Subantarctic fur seal females with two pups spent

less time ashore (Georges *et al.* 1999) while Antarctic fur seal females with two pups showed no difference in attendance or foraging-trip duration than females with single pups (Doidge 1987). In contrast, *A. forsteri* females with two pups on Kangaroo Island, Australia, spent more time ashore and less time at sea (Haase 2007).

During the 2003/04 breeding season at Ohau Point, a large New Zealand fur seal colony (producing ~450 pups that year), two cases of a female rearing two pups were observed. These cases were followed longitudinally to weaning. Maternal presence was recorded, the pups' growth was detailed using morphometry, and the relatedness between mothers and pups was examined using microsatellite genotyping. We discuss these cases, considering the costs of fostering in *A. forsteri*, with reference to their occurrence and frequency in otariids and other pinnipeds.

Materials and methods

Study site

This study was carried out during the 2003/04 austral summer at the Ohau Point seal colony, 25 km north of Kaikoura (42°15'S, 173°50'E), on the east coast of the South Island, New Zealand. The colony is ~1 km long, adjacent to State Highway 1, and primarily made up of large boulders and caves. The Kaikoura Canyon lies ~2 km offshore, providing a nearby access to a pelagic food source. Ohau Point was only recently recolonised and has expanded from 100 to nearly 600 pup births per year since 2000 (Boren *et al.* 2006).

Behavioural observations and measurements of pups

In 2004, 170 pups were marked using a combination of techniques including: haircuts (number patterns cut in guard hairs), Allflex® (NZ) sheep ear-tags, or yellow numbered caps glued to the fur on the back, posterior to the shoulder blades. Pups were caught by hand or with a noose, and weighed in a fabric sack from a Salter spring balance (20 × 0.2 kg). Dorsal standard length and axillary girth were measured.

Focal animal observations were carried out on both observed triads and on singleton pups for comparison. Instantaneous scan sampling (Altmann 1974) of known pups was carried out at 15-min intervals in a subsection of the breeding colony. Behaviours of interest included presence or absence of an adult female, association with the female, nursing, and time the pup spent on its own.

Genetic sampling and relatedness

DNA samples were obtained from females and pups to determine relatedness. Pups were caught and skin biopsies obtained from the trailing edge of fore flippers with piglet ear-notch pliers (Majluf and Goebel 1992). Hair samples were obtained from females using a modified crossbow with an adhesive dart to collect hair follicles from which DNA was extracted (Caudron *et al.* 2007). In addition to the samples collected from two mother–pups triads, samples from a further 157 individuals ($n = 20$ adult males, 46 adult females and 91 pups in 2003/04) were obtained as part of a longer-term study at Ohau Point. Samples were stored and processed as described by Caudron *et al.* (2007).

Nine informative microsatellite loci (HI16, Hg4.2, Hg6.1, Hg6.3, Pv9, Pv11, Lc5, M11a, 3E3) were used to derive individual genotypes, as described in Robertson and Gemmell (2005) and Negro *et al.* (2006). Duplicates from samples of the two female–pups triads were also run on an ABI 3100 sequencer (Applied Biosystems) to confirm the genotypes obtained.

The genotypes of the 157 individuals were used to derive a distribution of relatedness for the colony. The level of similarity between genotypes was calculated using the Queller and Goodnight relatedness estimator R (Queller and Goodnight 1989), as described in Gemmell (2003), with the R values derived using the program GenAlEx version 6 (Peakall and Smouse 2006). To determine the relatedness values associated with the total study population and known first-order relatives (mother–pups), we derived a distribution of R by making pair-wise genotypic comparisons of 157 individuals selected from the study area and 39 known mother–pup pairs respectively. We could then estimate genetic relatedness in the two cases of mother–pups triads.

Statistical analyses

Statistical analyses were carried out in Microsoft Office Excel 2003 (Microsoft Corporation), Minitab Release 14 (Minitab Inc.) and R v2.5.0 2007 (The R Foundation for Statistical Computing). To account for birth date and age at time of capture, growth rates were calculated longitudinally for all known-age pups ($n = 38$) using linear regression in R. Pupping at Ohau Point occurs over approximately a six-week period (Boren 2005). Birth date was estimated for 111 pups that could be classified as being born during pre-peak (18 November – 1 December), peak (2–15 December), or post-peak (16–31 December) pupping on the basis of the condition of their umbilicus on the date of first capture. Growth was then tested for differences between the sexes and timing of birth using ANCOVA in R.

To examine whether observed mass and growth differed between pups reared by females provisioning for two pups and pups reared by females provisioning for a single pup, one-sample t -tests were used (Zar 1999; Haase 2007). Values from pups of females rearing two pups that fell outside the 95% confidence interval of the mean of all other measured pups for their sex were considered to be significantly different.

The proportion of time spent nursing, in association with mother and the total times with and without mother were calculated relative to the total number of daily observations for each pup. All proportions were arcsine-transformed before statistical testing (Zar 1999). Grand means for all female and male singleton pups were calculated for each variable and one-sample t -tests were used to test for differences in nursing and attendance times between 'twins' and all the singleton pups.

Results

Maternity assignments

The estimate of genetic relatedness (R) calculated for 39 known mother–pup pairs was 0.472 (± 0.040 , 95% confidence interval), compared with the expected R value for first-degree relatives (mother and filial pup, full-siblings) of 0.5. The background distribution of relatedness in the population ($n = 157$) was esti-

Table 1. Genotypes at nine microsatellite loci for female–pup pair triads
Genotypes that are the same between mother and pups are shown in bold. MG, missing genotype

	Hg6.1	HI16	Lc5	Hg4.2	Pv11	M11a	Hg6.3	Pv9	3E3
Case 1									
Filial pup 1	152	155	159	165	155	145	236	172	216
	158	155	163	183	169	149	236	182	218
Filial pup 2	152	155	161	165	155	145	236	172	216
	154	155	163	169	169	177	236	182	220
Female A	152	155	159	165	155	145	236	170	218
	152	161	161	169	155	181	240	172	220
Case 2									
Filial pup 1	MG	141	161	137	155	145	234	172	216
	MG	149	165	137	163	159	242	172	216
Foster pup 2	MG	149	163	165	161	145	236	172	216
	MG	149	165	171	169	179	238	178	218
Female B	148	141	161	137	155	143	234	172	216
	154	153	165	163	155	145	236	172	216

mated at a mean R value of -0.006 (± 0.0033 , 95% confidence interval), which was not significantly different from zero.

For the two cases where a female actively suckled two pups through the course of the normal lactation period, genotypic analyses confirmed two triads, one comprising a female, a filial pup and a foster pup, and the other comprising a female and two filial pups (Table 1). In the former case no mismatches were found between the female and Pup 1 ($r = 0.614$), showing that these individuals were first-order relatives; however, the female and Pup 2 were found to be unrelated, with several mismatches found between them and an R value of -0.099 (Fig. 1). On the basis of the estimate of relatedness for first-order relatives at Ohau Point (0.472) we would expect third-order relatives (i.e. first cousins) to have an R value of ~ 0.118 . The pairwise R estimate between Pup 1 and Pup 2 in this case is 0.093, similar to the estimate of first cousins.

In the case where the female nursed two filial pups (twins) no mismatches were found between the female and each pup or between the pups. The pairwise relatedness values between individuals of the triad fall within the distribution of first-order relatives, with the pups being full siblings ($r = 0.684$) (Fig. 1). The pairwise R estimate between the female and Pup 1 ($r = 0.340$) was lower than the pairwise R estimate between the female and Pup 2 ($r = 0.491$), mainly because many alleles shared between the female and Pup 1 were common alleles in the population.

Twining (Case 1)

The twin pups were discovered on 22 December 2003. Both pups were estimated at one day old or less, being born after the breeding peak. The twins were observed nursing at the same time on numerous occasions; however, the male twin (X7) was often successful in excluding the smaller female twin (X6) from access to a teat.

For known-age pups ($n = 38$) there was a slight difference in growth depending on sex (females: 62.6 g day^{-1} ; males: 73.2 g day^{-1} ; $P = 0.0214$) and approximate date of birth (pre-peak: 71.4 g day^{-1} ; peak: 71.5 g day^{-1} ; post-peak: 56.7 g day^{-1} ; $P = 0.0240$); however, with the larger sample size of 111 pups, minimal difference was detected in sex (females: 64.0 g day^{-1} ; males 70.4 g day^{-1} ; $P = 0.0521$) and none detected for date of

birth (pre-peak: 72.2 g day^{-1} ; peak: 67.9 g day^{-1} ; post-peak: 63.5 g day^{-1} ; $P = 0.133$). Accounting for age, both twins were significantly lighter than other pups of the same sex ($n = 38$); however, only X6 was significantly lighter at birth (Fig. 2). Both X6 and X7 had exhibited significantly slower growth from birth to approximately three months of age (36.5 versus 62.6 g day^{-1} , $P < 0.001$ and 39.9 versus 73.2 g day^{-1} , $P < 0.001$). Their growth was still lower than average compared with just the post-peak pupping age group (63.5 g day^{-1} , $n = 22$ estimated-age pups). X6 and X7 weighed 6.4 and 8.8 kg, respectively, at 10 months of age.

X6 spent a significantly lower proportion of time nursing and with her mother than singleton pups did. This was not the case for X7 (Fig. 3). X7 was last observed nursing from his mother at 11 months old. At 12 months, he would sit close to her and her newborn but the female made open-mouth threats

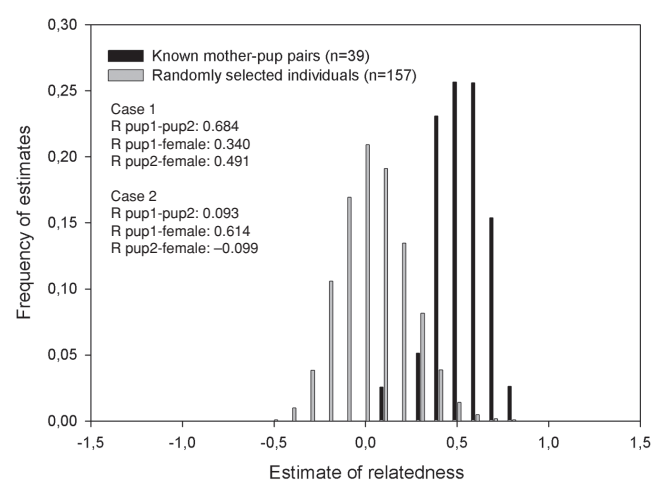


Fig. 1. Distribution of estimated pairwise relatedness among known mother–pup pairs and among the randomly selected individuals to determine the background distribution of relatedness in the population (12245 pairwise relatedness estimates). Case 1 is the triad comprising the female and the filial pup pair, and Case 2 is the triad comprising the female, the filial pup and the foster pup.

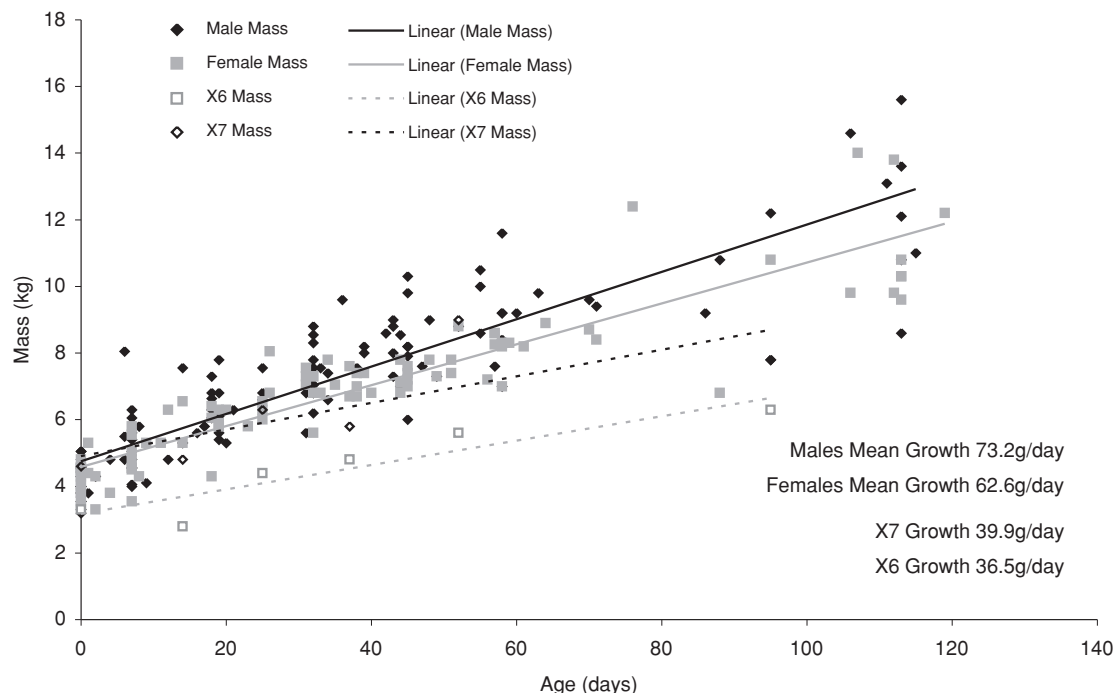


Fig. 2. Mass of twins X6 and X7 compared with mean values for female and male pups, respectively, of the same age.

when he attempted to nurse. After 10 months of age X6 was never seen nursing from her mother although she successfully stole milk from other females. The twins were last seen at just over 12 months of age and were the last pups to leave the colony with the exception of one that was nursed for two years. X7 was emaciated while X6 appeared in a better, but still poor,

condition. It is not known whether they survived into the following season.

Fostering (Case 2)

One case of long-term fostering of an additional pup was observed in the 2004 season, out of 451 pups (Boren *et al.*

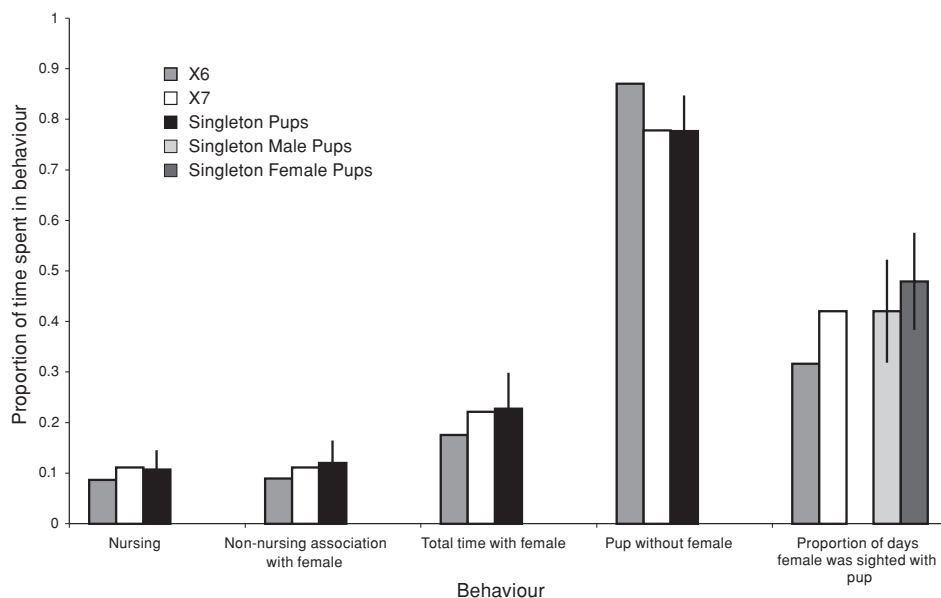


Fig. 3. Mean nursing and attendance proportions from the pups' perspective for the twins X6 and X7 compared with the average (error bars given as the confidence interval of the mean) for singleton pups. Data for female and male pups ($n = 42$) were pooled for the first four variables but not the female's proportion of time in attendance as they were significantly different (females: $n = 17$; males: $n = 23$).

2006), resulting in a minimum frequency of fostering of 0.22% for the year. In this case, two pups were observed together with a female on six occasions between 8 December 2003 and 27 April 2004. The female was not identifiable and only one of the pups was marked on 30 January 2004. Before this date it was assumed that these were the same individuals as they were always in the same location and it was considered unlikely that there would be more than one occurrence of fostering in the same area. This triad was first observed on 8 December 2003, when two pups were seen resting next to a female; the female looked at both pups and made naso-nasal contact with the closest pup and no rejection of either pup. This triad was observed again undertaking similar behaviours on five other occasions from 9 December 2003 to 27 April 2004.

Both male pups appeared to be of similar weight and condition on all observations except on 27 April 2004 when the non-filial pup was in visibly poor condition. The first day the triad was observed was three days after the median pupping date (5 December) and they were of a similar size to other pups born during peak pupping. To account for differences in weight that could be a result of age at the time of weighing the filial pup was compared with other male pups born during peak pupping ($n = 46$ estimated-age pups). The filial pup was initially heavier than other male pups born during peak pupping and weighed on 30 January (9.8 versus 8.9 ± 0.23 kg, $n = 32$), but on 26 March he weighed more than 1 kg less than other male pups (10.6 versus 11.9 ± 0.32 kg, $n = 29$). His growth from January to March was also outside the 95% confidence interval of the mean (14.3 versus 54.5 ± 4.7 g day⁻¹, $n = 22$).

Discussion

Potential costs of provisioning two pups

As expected, mass and growth of the twin pups were significantly lower than for singleton pups of the same sex throughout the year (Fig. 2). The male twin did not differ in birth mass from singleton pups, which indicates that he was not disadvantaged by the multiple births but rather did not gain sufficient nutrition during lactation due to their mother providing for two pups. This is consistent with a study on southern elephant seal (*Mirounga leonina*) twins that found mean birth masses did not differ between twin and singleton pups but that twins grew more slowly (McMahon and Hindell 2003). Lower growth rates (Doidge 1987) and weaning weights (Doidge 1987; Georges *et al.* 1999) were observed in otariid pup dyads, as well as reduced body condition (Haase 2007).

The time the twins' mother spent nursing and in association with X7 was not significantly different from that of other mother-pup pairs but she spent significantly less time nursing and in association with X6 (Fig. 3). She continued to nurse the male twin (X7) later into the season, which may have been to compensate for a lack of milk provisioned. This is consistent with a study by Georges *et al.* (1999), which found that the biological pup of a filial/non-filial dyad reared by one mother weaned later than singleton pups of the same cohort. There is probably a maximum amount of maternal resource available to a female to provision her pup, and perhaps females normally care for single pups near this metabolic maximum (Arnbom *et al.* 1997). While the twins' mother did provision the pups sufficiently for them to

survive to weaning, their probability of surviving to breeding was likely to be low (on the basis of their body condition at last sighting). This suggests that the female was probably provisioning the twins near her physiological maximum without jeopardising her own survival or future reproductive success.

A similar outcome was found for the fostering triad, where the filial pup was of a significantly lower mass and had a lower growth rate than other male pups raised alone. The fostered pup was in a visually worse condition than the filial pup. Potential costs to the foster mother are more difficult to discuss as she was not easily identifiable when not with her pups. However, she may have reduced her reproductive success by directing some nutritional resources to the non-filial pup instead of focusing only on her biological offspring, which resulted in the filial pup being lighter and exhibiting a slower growth rate than singleton pups.

Possible causes of fostering

Unfortunately, we could not determine the cause of fostering. The case was first recorded when the pups were up to two weeks old, which suggests that it may have occurred before mother-pup recognition was fully developed, possibly caused by a disturbance in the colony or high density at the pupping site (Fogden 1971; Stirling 1975). The propensity of *A. forsteri* females to return to give birth at their natal colony (Stirling 1971) and the recent recolonisation of the Ohau Point colony (Boren *et al.* 2006) suggest that relatedness among females may be high. Additionally, the relatedness estimate of 0.093 for the foster pups provided evidence that they may be first cousins. Therefore, the hypothesis relating kin selection to fostering cannot be ruled out. Maternal experience may also have been a factor, although the age of the female was unknown, since the fostering occurred in a new pupping area in the colony (2002–03) where younger females tend to colonise (Goldsworthy and Shaughnessy 1994).

Conclusions

Until recently, twinning was thought to be nonexistent or extremely rare in otariids (Marlow 1972; Stirling 1975). Fostering has been observed in most species of otariids but generally at lower frequencies than in phocids. The case of twins found during this study is only the third known occurrence in *A. forsteri* (Miller 1971; Armstrong 1988). The frequency of fostering we found (0.22%) for the 2003/04 season is similar to the 0.17% frequency found in a study of *A. forsteri* in Australia (Haase 2007), but is considerably lower than those found in *A. gazella* (7%: Lunn 1992; 11%: Gemmell 2003) and New Zealand sea lions, *Phocarcus hookeri* (6%: Childerhouse and Gales 2001).

Acknowledgements

The authors thank Melinda Fowler, Olivier Biémar, Leona Laniewa, John Klavitter, Catherine Gunther-Murphy and the Canterbury University Marine Ecology Research Group for assistance in the field. The New Zealand Department of Conservation provided logistical assistance. Financial assistance was provided by the Minority International Research Training Program, a Canterbury University Doctoral Scholarship and the Whale and Dolphin Adoption Project. Research was carried out under Department of Conservation Permit: Per/10/2002/01. Two anonymous reviewers provided helpful comments on the manuscript.

References

- Altmann, J. (1974). Observational study of behaviour: sampling methods. *Behaviour* **49**, 227–265. doi:10.1163/156853974X00534
- Armstrong, M. (1988). Census and some behavioural and thermoregulatory studies of the fur seal, *Arctocephalus forsteri* (Lesson 1828), on the Otago Peninsula, New Zealand. M.Sc. Thesis, University of Otago, Dunedin.
- Arnbom, T., Fedak, M. A., and Boyd, I. L. (1997). Factors affecting maternal expenditure in southern elephant seals during lactation. *Ecology* **78**, 471–483.
- Bester, M. N., and Kerley, G. I. H. (1983). Rearing of twin pups to weaning by subantarctic fur seal *Arctocephalus tropicalis* female. *South African Journal of Wildlife Research* **13**, 86–87.
- Boness, D. J., Bowen, D., Iverson, S. J., and Oftedal, O. T. (1992). Influence of storms and maternal size on mother–pup separations and fostering in the harbour seal, *Phoca vitulina*. *Canadian Journal of Zoology* **70**, 1640–1644.
- Boren, L. J. (2005). New Zealand fur seals (*Arctocephalus forsteri*) in the Kaikoura region: colony dynamics, maternal investment and health. Ph.D. Thesis, University of Canterbury, Christchurch.
- Boren, L. J., Muller, C. G., and Gemmell, N. J. (2006). Colony growth and pup condition of the New Zealand fur seal (*Arctocephalus forsteri*) on the Kaikoura coastline compared with other east coast colonies. *Wildlife Research* **33**, 497–505. doi:10.1071/WR05092
- Bryden, M. M. (1966). Twin fetuses in the southern elephant seal, *Mirounga leonina* (L.). *Papers and Proceedings of the Royal Society of Tasmania* **100**, 89–90. doi:10.1111/j.1748-7692.2006.00084.x
- Caudron, A. K., Negro, S. S., Muller, C. G., Boren, L. J., and Gemmell, N. J. (2007). Hair sampling and genotyping from follicles: a minimally-invasive alternative for genetics studies in small, mobile pinnipeds. *Marine Mammal Science* **23**, 184–192. doi:10.1111/j.1748-7692.2006.00084.x
- Childerhouse, S., and Gales, N. (2001). Fostering behaviour in New Zealand sea lions *Phocartos hookeri*. *New Zealand Journal of Zoology* **28**, 189–195.
- Clutton-Brock, T. H., Albon, S. D., and Guinness, F. E. (1989). Fitness costs of gestation and lactation in wild mammals. *Nature* **337**, 260–262. doi:10.1038/337260a0
- Dobson, F. S., and Jouventin, P. (2003). How mothers find their pups in a colony of Antarctic fur seals. *Behavioural Processes* **61**, 77–85. doi:10.1016/S0376-6357(02)00164-X
- Doidge, D. W. (1987). Rearing of twin offspring to weaning in Antarctic fur seals, *Arctocephalus gazella*. US Department of Commerce, NOAA Technical Report NMFS 51.
- Fay, F. H., Burns, J. J., Kibal'chich, A., and Hills, S. (1991). Incidence of twin fetuses in walrus (*Odobenus rosmarus* L.). *Northwestern Naturalist* **72**, 110–113. doi:10.2307/3536496
- Fogden, S. C. L. (1971). Mother–young behaviour at grey seal breeding beaches. *Journal of the Zoological Society, London* **164**, 61–92.
- Gelatt, T. S., Davis, C. S., Siniff, D. B., and Strobeck, C. (2001). Molecular evidence for twinning in weddell seals (*Leptonychotes weddellii*). *Journal of Mammalogy* **82**, 491–499. doi:10.1644/1545-1542(2001)082<0491:MEFTIW>2.0.CO;2
- Gemmell, N. J. (2003). Kin selection may influence fostering behaviour in Antarctic fur seals (*Arctocephalus gazella*). *Proceedings of the Royal Society of London. Series B. Biological Sciences* **270**, 2033–2037. doi:10.1098/rspb.2003.2467
- Georges, J. Y., Sevot, X., and Guinet, C. (1999). Fostering in a subantarctic fur seal. *Mammalia* **63**, 384–388.
- Goldsworthy, S. D., and Shaughnessy, P. D. (1994). Breeding biology and haul-out pattern of the New Zealand fur seal, *Arctocephalus forsteri*, at Cape Gantheaume, South Australia. *Wildlife Research* **21**, 365–376. doi:10.1071/WR9940365
- Haase, T. J. (2007). Suckling an additional pup to weaning in the New Zealand fur seal. *Marine Mammal Science* **23**, 176–183. doi:10.1111/j.1748-7692.2006.00078.x
- Hamilton, W. D. (1964). The genetic theory of social behaviour I, II. *Journal of Theoretical Biology* **7**, 1–52. doi:10.1016/0022-5193(64)90038-4
- Insley, S. J. (2001). Mother–offspring vocal recognition in northern fur seals is mutual but asymmetrical. *Animal Behaviour* **61**, 129–137. doi:10.1006/anbe.2000.1569
- Ling, J. K. (1986). Twin fetuses from an Australian sea lion, *Neophoca cinerea* (Carnivora: Otariidae). *Australian Mammalogy* **9**, 9–60.
- Lunn, N. J. (1992). Fostering behaviour and milk stealing in Antarctic fur seals. *Canadian Journal of Zoology* **70**, 837–839.
- Majluf, P., and Goebel, M. E. (1992). The capture and handling of female South American fur seals and their pups. *Marine Mammal Science* **8**, 187–190. doi:10.1111/j.1748-7692.1992.tb00382.x
- Marlow, B. J. (1972). Pup abduction in the Australian sea lion, *Neophoca cinerea*. *Mammalia* **36**, 161–165.
- McMahon, C. R., and Hindell, M. A. (2003). Twinning in southern elephant seals: the implications of resource allocation by mothers. *Wildlife Research* **30**, 35–39. doi:10.1071/WR01069
- Miller, E. H. (1971). Social and thermoregulatory behaviour of the New Zealand fur seal, *Arctocephalus forsteri* (Lesson, 1828). M.Sc. Thesis, University of Canterbury, Christchurch.
- Negro, S. S., Cloots, R., and Gemmell, N. J. (2006). Cost-effective media for the rapid and high resolution of small DNA fragments using polyacrylamide-based electrophoresis. *Molecular Ecology* **6**, 609–612. doi:10.1111/j.1471-8286.2006.01290.x
- Oftedal, O. T., Boness, D. J., and Tedman, R. A. (1987). The behaviour, physiology, and anatomy of lactation in the Pinnipedia. *Current Mammalogy* **1**, 175–245.
- Peakall, R., and Smouse, P. (2006). Genalex6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology* **6**, 288–295. doi:10.1111/j.1471-8286.2005.01155.x
- Phillips, A. V. (2003). Behavioural cues used in reunions by South American fur seals, *Arctocephalus australis*. *Journal of Mammalogy* **84**, 524–535. doi:10.1644/1545-1542(2003)084<0524:BCUIRB>2.0.CO;2
- Queller, D. C., and Goodnight, K. F. (1989). Estimating relatedness using genetic markers. *Evolution* **43**, 258–275. doi:10.2307/2409206
- Rae, B. B. (1969). Twin seals in Scotland. *Journal of Zoology* **158**, 243–245.
- Riedman, M. L. (1982). The evolution of alloparental care and adoption in mammals and birds. *The Quarterly Review of Biology* **57**, 405–435. doi:10.1086/412936
- Riedman, M. L. (1990). 'The Pinnipeds: Seals, Sea lions and Walrusen.' (University of California Press: Berkeley, CA.)
- Riedman, M., and Le Boeuf, B. J. (1982). Mother–pup separation and adoption in northern elephant seals. *Behavioral Ecology and Sociobiology* **11**, 203–215. doi:10.1007/BF00300063
- Robertson, B. C., and Gemmell, N. J. (2005). Microsatellite DNA markers for the study of population structure in the New Zealand fur seal (*Arctocephalus forsteri*) CSL Contract 3053. Department of Conservation, Wellington, New Zealand.
- Roulin, A. (2002). Why do lactating females nurse alien offspring? A review of hypotheses and empirical evidence. *Animal Behaviour* **63**, 201–208. doi:10.1006/anbe.2001.1895
- Roulin, A. (2003). The neuroendocrine function of allosuckling. *Ethology* **109**, 185–195. doi:10.1046/j.1439-0310.2003.00870.x
- Schaeff, C. M., Boness, D. J., and Bowen, W. D. (1999). Female distribution, genetic relatedness, and fostering behaviour in harbour seals, *Phoca vitulina*. *Animal Behaviour* **57**, 427–434. doi:10.1006/anbe.1998.1001
- Spotte, S. (1982). The incidence of twins in pinnipeds. *Canadian Journal of Zoology* **60**, 2226–2233.
- Stirling, I. (1971). Studies on the behaviour of the South Australian fur seal, *Arctocephalus forsteri* (Lesson). II. Adult females and pups. *Australian Journal of Zoology* **19**, 267–273. doi:10.1071/ZO9710267

- Stirling, I. (1975). Adoptive suckling in pinnipeds. *Journal of the Australian Mammal Society* **1**, 389–391.
- Trillmich, F. (1981). Mutual mother–pup recognition in Galapagos fur seals and sea lions: cues used and functional significance. *Behaviour* **78**, 21–42. doi:10.1163/156853981X00248
- Vania, J. S. (1965). Marine mammal investigations: sea lions. Federal Aid in Wildlife Restoration Annual Project Segment Report. Alaska Fish and Game. Juneau, Alaska.
- Zar, J. H. (1999). 'Biostatistical Analysis.' (Prentice-Hall Inc.: Upper Saddle River, NJ.)

Manuscript received 29 November 2007, accepted 21 May 2008